PROTECTIVE EFFECT OF *Desmostachya bipinnata (L.) Stapf* AGAINST PARACETAMOL-INDUCED HEPATIC DAMAGE

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Certificate

This is to certify that the dissertation entitled "**PROTECTIVE EFFECT OF *Desmostachya bipinnata* (L.) Stapf AGAINST PARACETAMOL-INDUCED HEPATIC DAMAGE"** being submitted to The Tamil Nadu Dr.M.G.R. Medical University, Chennai in partial fulfillment of the Master of Pharmacy programme in Pharmacology, carried out by **INDRASENAN. R**, in the Department of Pharmacology, College of Pharmacy, SRIPMS, Coimbatore, under my direct guidance and supervision to my fullest satisfaction.

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Indrasenan. R

CONTENTS

LIST OF ABBREVIATIONS

ALP	:	Alkaline phosphatase
ALT	:	Alanine transaminase
AST	:	Aspartate transaminase
CAT	:	Catalase
CF	:	Chloroform fraction
EAF	:	Ethyl acetate fraction
GSSH	:	Glutathione reductase
GSH	:	Reduced glutathione
GPx	:	Glutathione peroxidase
LH	:	Lipid hydroperoxides
LDH	:	Lactate Dehydrogenase.
MDA	:	Malondialdehyde
mg/dl	:	milligram/deciliter
MPT	:	Mitochondrial permeability transition
nBF	:	n-Butanol fraction

PcmL	:	Paracetamol
PEF	:	Petroleum ether fraction
Px	:	Peroxidase
RNS	:	Reactive Nitrogen Species
ROS	:	Reactive Oxygen Species
rpm	:	Rotation per minute
SGOT	:	Serum oxaloacetate transaminase
SGPT	:	Serum glutamate pyruvate transaminase
SOD	:	Superoxide dismutase
TBA	:	Thiobarbituric acid
TBARS	:	Thiobarbituric acid reactive substances
TCA	:	Trichloroacetic acid
TP	:	Total Protein
U/L	:	Units/liter
µg	:	Microgram

1. INTRODUCTION

LIVER

Liver is the largest gland in the body weighing between 1 to 2.3 kg. It is situated in the upper part of the abdominal cavity occupying the greater part of the right hypochondriac region. The liver consists of 4 lobes, a large right lobe and the smaller wedge shaped left lobe. The other two, the caudate and quadrate lobes are areas on the posterior surfaces (Waugh and Grant, 2001).

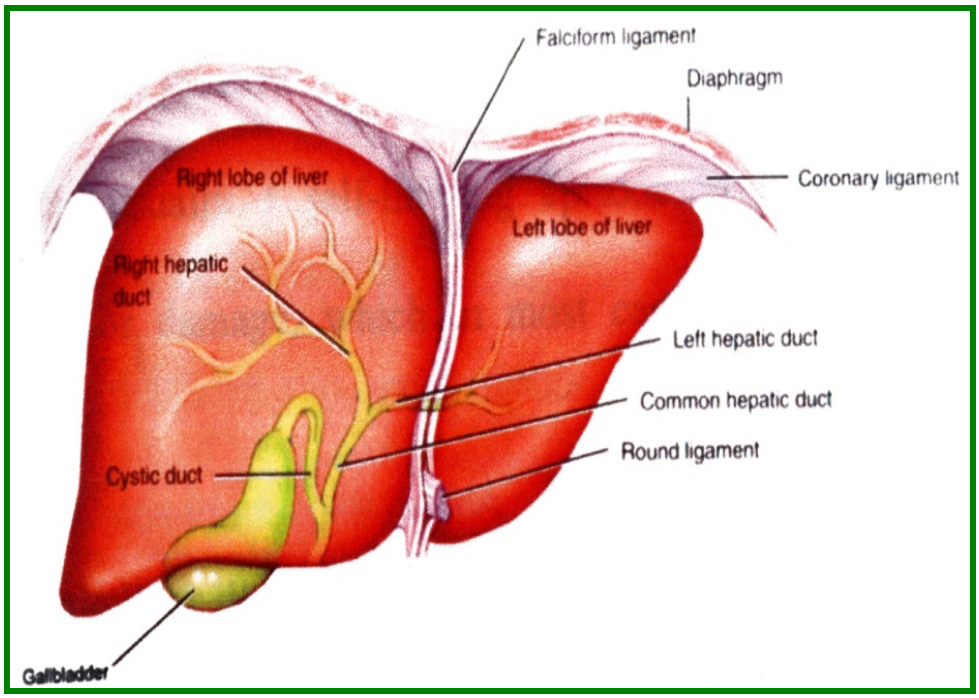


Fig. 1 Anterior view of liver (Tortora and Derrickson, 2006)

Organs associated with liver

Superiorly & anteriorly : Diaphragm and abdominal wall.

Inferiorly : Stomach, bile ducts, duodenum, right kidney.

Posteriorly : Oesophagus, inferior venecava, aorta, gall bladder.

Laterally : Lower ribs and diaphragm.

Anatomy of the liver

The liver is almost completely covered by visceral peritoneum and by a dense irregular connective tissue layer that lies deep to the peritoneum. The right and left lobes of the liver are separated by the falciform ligament- a fold of peritoneum. The falciform ligament extends from the under surface of the diaphragm between the two principal lobes of the liver to the superior surface of the liver helping to suspend the liver in the abdominal cavity. The right and left coronary ligaments are narrow extension of the parietal peritoneum that suspends the liver from diaphragm (Tortora and Derrickson, 2006).

Histology of the liver

The lobes of the liver are made up of many functional units called lobules. A lobule is typically a six sided (hexagon) structure that consists of specialized epithelial cells called hepatocytes, arranged in irregular, branching, inter connected plate around a central vein. In addition the liver lobule contains highly permeable capillaries called sinusoids through which blood passes. The fixed phagocytes called stellate reticulo endothelial cells (Kupfer cells) are also present in the sinusoids which destroy worn out white blood cells and red blood cell bacteria and other foreign matter in the blood draining from gastrointestinal tract (Tortora and Derrickson, 2006).

Blood supply to the liver

The liver receives blood from two sources. From the hepatic artery it obtains oxygenated blood and from the

hepatic portal vein it receives deoxygenated blood containing newly absorbed nutrients, drugs, and possibly microbes and toxins from the gastrointestinal tract (Tortora and Derrickson, 2006).

Secretions of liver

Bile is both a product of secretion as well as an excretion of the liver (Chaterjee, 2004).

Composition of bile

Total quantity : 500-1000 ml daily.

Colour : Yellowish green.

Taste : Bitter.

Bile salts : Sodium taurocholate and sodium glycocholate.

Bile pigments : Bilirubin and biliverdin.

Inorganic salts : Chlorides, carbonates.

Functions of the liver (Waugh and Grant, 2001).

i) Deaminates amino acids

ii) Converts glucose to glycogen

iii) Desaturates fat

iv) Produce heat

v) Secretes bile

vi) Synthesize vitamin A, non essential amino acids, plasma proteins and blood clotting factors.

vii) Detoxicates drugs and noxious substances

viii) Metabolizes ethanol

ix) Inactivates hormones

x) Stores fat-soluble and water- soluble vitamins, iron and copper.

Liver diseases

There are two types of liver diseases, namely acute

and chronic,

a) Acute liver disease

This is usually a self-limiting episode of liver cell (hepatocyte) inflammation or damage, which in most cases resolves without clinical sequel.

b) Chronic liver disease

This occurs when permanent structural changes within the liver occur secondary to long- standing cell damage, with the consequent loss of normal liver architecture. In most cases, this progress to cirrhosis and leads to liver failure.

Causes of liver diseases

i) Viral infections-Hepatitis A, B, C, D, E

ii) Alcohol

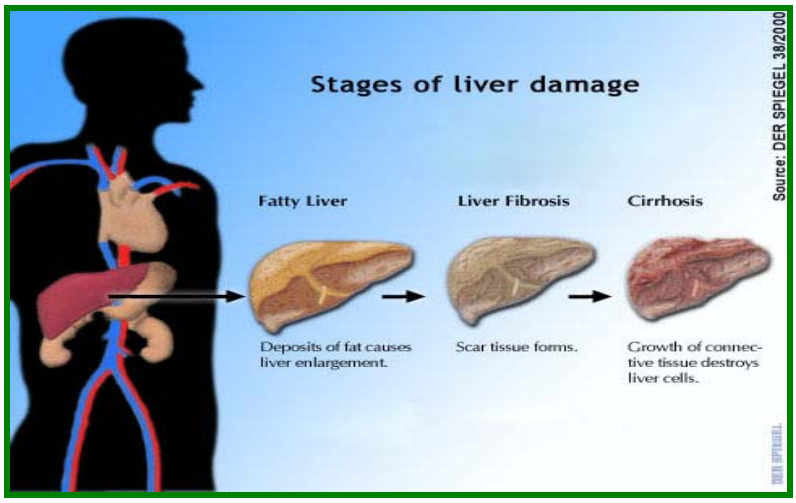


Fig. 2. Stages of liver damage

The ingestion of ethanol in alcoholic beverages is the commonest cause of liver cirrhosis. Prolonged and excessive exposure to alcohol induces inflammatory activity within the liver tissue and the hepatocytes accumulate large droplets of fat as inclusion bodies within the swollen cells. A fine network of collagen fibers develop around the liver cells near the hepatic venule, and the extent of fibrosis increases which eventually leads to liver failure.

iii) Immune disorders

Autoimmune diseases occurs when the immune system develops autoantibodies within the body. e.g. Autoimmune hepatitis.

iv) Vascular abnormalities

The Budd-Chiare syndrome occurs when obstruction of the major hepatic veins leads to cell destruction and cirrhosis. Veno-occlusive disease occurs when the smaller veins of liver become obliterated as a result of exposure to toxins, irradiation or cytotoxicity.

MARKERS IN LIVER DISEASE

Alanine Transaminase (ALT)

ALT is an enzyme produced in the hepatocytes. This enzyme functions normally to transfer the amino group from an aminoacid alanine to a keto acid producing pyruvate. The activities of ALT outside the liver are low and therefore this enzyme is considered more specific for

hepatocellular damage. As cells are damaged, ALT leaks out into the bloodstream and the levels are elevated in cases of hepatitis, shock or drug toxicity. It is a measure of integrity of cell death or inflammation (Harrison, 1990).

Aspartate Transaminase (AST)

It is an enzyme similar to ALT and is also produced in the muscle. This enzyme transfers the amino group from the amino acid aspartate to a keto acid producing oxaloacetate. It can be elevated in other conditions like heart attack. In case of liver inflammation, the ALT and AST activities are elevated in the ratio of 1:1. In certain conditions like alcoholic or shock liver, the level of AST is more elevated than ALT.

Alkaline Phosphatase (ALP)

Alkaline phosphatase is a group of enzymes that are capable of hydrolyzing phosphate esters at alkaline pH and are widely distributed in the body with significant

activities in the liver, GIT, bone and placenta. These enzymes are found in greatest concentration in membranes associated with absorption and secretory functions. In the liver they are localized in the sinusoidal and biliary canalicular membrane.

Gama glutamyl transferase (GGT)

It is a microsomal enzyme found in many cells and tissues of the body. The largest concentrations are found in the liver, localized in the hepatocytes and epithelium of the small bile ducts.GGT functions normally to transfer glutamyl groups from gama glutamyl peptides to other peptides and amino acids.

Lactate dehydrogenase (LDH)

Lactate dehydrogenase is an enzyme catalyzing oxidation of lactic acid to pyruvic acid. In blood serum there are five physically distinct iso enzymes of this enzyme. They are known as LDH 1-5. All these iso enzymes though different, physically catalyse the same reaction of

oxidation of lactic acid to pyruvic acid. If LDH4 and LDH5 are increased it is a case of liver disease.

Bilirubin

Bilirubin is the breakdown product from the destruction of old red lood cells. The level of bilirubin is elevated in the blood either by increased production, decreased uptake by the liver, decreased conjugation and decreased secretion from liver or blockage of the bile ducts. Increased production produces unconjugated or indirect bilirubin and decreased production produces conjugated or direct bilirubin.

HEPATOTOXICITY

Liver toxicity is a major health problem of worldwide proportions. It is influenced by various physiological, nutritional and therapeutic factors. It can be modulated when exposure occurs in the presence of enzyme inducers. Drug induced liver injury appears to involve two

pathways,

1) Direct hepatotoxicity

2) Adverse immune reactions

In most cases, it is initiated by the activation of drugs to chemically reactive metabolites, which have the ability to interact with cellular macromolecules like proteins, lipids and nucleic acids causing protein dysfunction, lipid peroxidation, DNA damage and oxidative stress, thus leading to the impairment of cellular function which can culminate in liver failure and cell death (Zhang, 2002).

CLASSIFICATION OF HEPATOTOXIC AGENTS

The hepatotoxic agents can be broadly classified into two major categories (Zhang, 2002).

1) True, intrinsic or predictable hepatotoxins:

Their hepatotoxicity is a fundamental property to which most exposed individuals are susceptible.

2) Non-predictable or idiosyncratic hepatotoxins:

Consist of agents that produce hepatic injury only in unusually susceptible humans i.e., their toxic effect results from the special vulnerability of the affected individual.

Table 1. CLASSIFICATION OF HEPATOTOXIC AGENTS

Category of Agents	Mechanism	Histological Lesion	Examples
I)Intrinsic toxins a) Direct	Direct physiochemical disortion and destruction of structural basis cell metabolism	Necrosis (zonal) and /or steatosis	CCl_4,$CHCl_3$, phosphorous, tetrachloroethane
b) Indirect i) Cytotoxic	Interference with specific metabolic pathways leading to structural injury	Steatosis or necrosis	Paracetamol, ethionine, ethanol, galactosamine, natural

			hepatotoxins
ii) Cholestatic	Interfere with hepatic excretory pathways leading to cholestasis	Bile casts	Icterogenin, anabolic and contraceptive steroids
II)Host idiosyncrasy a) Hypersensitivity b)Metabolic abnormality	Drug allergy Production of hepatotoxic metabolite	Necrosis/ cholestasis Necrosis or cholestasis	Sulphonamides, p-amino salicylic acid, halothane Iproniazoid, isoniazid, halothane

Drug induced liver injury (DILI)

Drug induced liver injury is intiated by direct hepatotoxic effect of a drug or a reactive metabolite of a drug. Parenchymal cell injury indicates activation of innate and or adaptive immune cells which in turn produce pro inflammatory and tissue hepatotoxic mediators and or mount immune reactions against drug associated antigens. DILI accounts for more than 50% of acute liver failure including hepatotoxicity caused by over dose of acetaminophen (APAP) 39% and idiosyncratic liver injury triggered by other drugs 13% (Holt, 2006).

MECHANISM OF DRUG INDUCED LIVER INJURY

Drug induced direct hepatotoxicity

In most instances DILI is intiated by the bioactivation of drugs to chemically reactive metabolite which have the ability to interact with cellular macro molecules such as proteins, lipids, and nucleic acids leading to proten

dysfunction, lipid peroxidation, DNA damage and oxidative stress. Additionally these reactive metabolite may induce disruptions of ionic gradients and intra cellular calcium stores resulting in mitochondrial dysfunction and loss of energy production. This impairement of cellular function can culminate in cell death and possible liver failure. DILI can affect both parenchymal and non parenchymal cells of the liver, leading to a wide variety of pathological conditions.

Role of innate immunity in DILI

Drug induced stress or damage of hepatocytes may trigger activation of inflammatory responses of the innate immune system with in liver. Evidence to support this idea has been mainly obtained from studies of liver injury induced by over dose of APAP. The intial NAPQI induced hepatocyte damage may lead to activation of innate immune cells with in the liver there by stimulating hepatic infiltration of inflammatory cells. Activated cells of the

innate immune system produce a range of inflammatory mediators including cytokines, chemokines, and reactive oxygen and nitrogen species that contribute to the progression of liver injury.

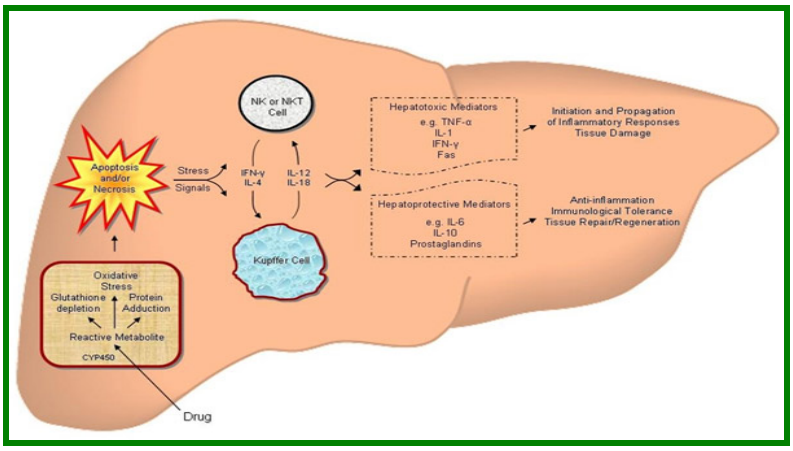


Fig. 3. Mechanism of Drug induced liver injury

The innate immune cells reported to participate in APAP hepatotoxicity include natural killer (NK), and natural killer T (NKT) cells, macrophages, and neutrophills. The depletion of NK and NKT cells protected mice from APAP induced liver injury. This protective mechanism seems to involve eliminating the production of IFN-γ and various other pro inflammatory chemokines as well as

decreasing neutrophil accumulation with in the liver.

APAP hepatotoxicity has also been attributed in part to the activation of Kupfer Cells (KC) secondary to hepatocyte damage. KC activation results in the release of a wide range of pro inflammatory mediators such as TNF a which may directly induce tissue damage and IL-12 and IL-18 which are important activation of NK and NKT cells.

KC may play a protective role in addition to their pro toxicant effect as KC are the pre dominant source of IL-10 and IL-16 which are important in counter acting inflammatory responses or stimulating liver regeneration.

PARACETAMOL (PcmL)

It is the active metabolite of phenacetin. PcmL is an effective alternative to aspirin as an analgesic and antipyretic, but a weak anti-inflammatory agent. Lesions produced by PcmL are due to glutathione depletion, chemical modification of critical cellular macromolecules

and oxidative stress. During PcmL intoxication both covalent binding to cellular macromolecules and lipid peroxidation occurs (Arnaiz *et al.*, 1995).

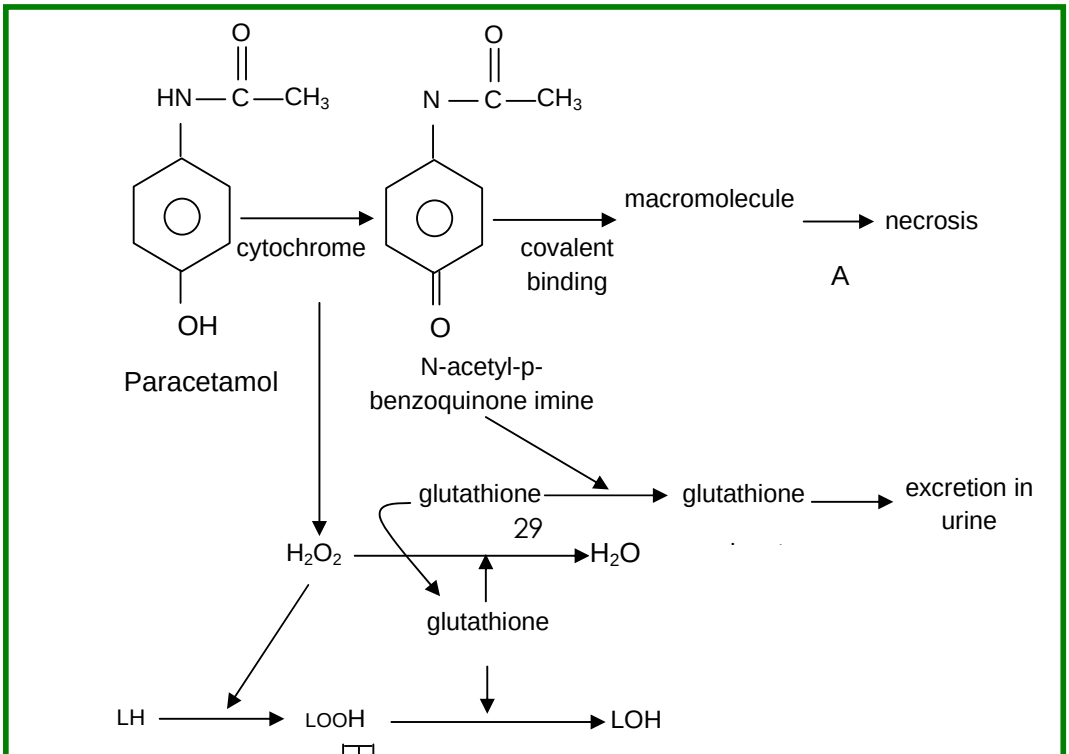
The active metabolite capable of binding covalently to proteins is found to be N-acetyl-p-benzoquinoneimine. It binds to proteins and also other nucleophiles. It contributes to overall process leading to necrosis (Gupta *et al.*, 2004).

PcmL is a mild analgesic that is harmless in therapeutic doses and potent a hepatotoxin in overdose. It has been proved to be an elegant model for the exploration of the mechanisms of hepatotoxicity (Sumioka *et al.*, 2004). PcmL is largely converted to conjugates of glucuronate and sulfate (Gupta *et al.*, 2004). A minor amount, about 25%, is converted to the active metabolite which binds promptly to glutathione (GSH), the resulting

compound being converted to mercaptopuric acid and cysteine. When the amount of active molecule formed exceeds the GSH available for binding, and then it causes necrosis.

PcmL causes hepatic necrosis only when the dose is sufficiently large to deplete hepatic GSH by 85% or more. Susceptibility to hepatotoxic effects relates to the rate of conversion of Pcml to an active metabolite, presumably a reactive imidoquinone derived from N-hydroxyl-N-acetyl-p-hydroxyaniline.

Fig. 4 Mechanisms involved in the hepatotoxic action of paracetamol

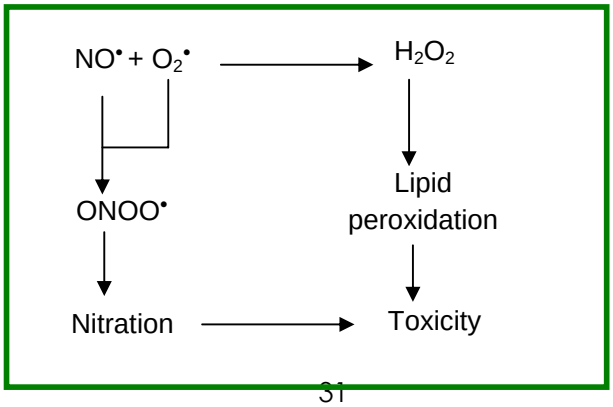


OXIDATIVE STRESS AND FREE RADICAL GENERATION

Oxidative stress occurs by acute paracetamol administration in mice liver. Paracetamol hepatotoxicity appears to be critically dependent on the depletion of cellular glutathione. A relatively high reduction in the intracellular level of reduced glutathione leads to oxidative stress. The one electron oxidation of paracetamol by P450 stepwise may generate reactive oxygen species, and the subsequent thiol depletion

through oxidation may lead to an alteration in calcium homeostasis and cause hepatotoxicity. It is suggested that an alkylating paracetamol metabolite cause Ca^{2+} de-regulation in the nucleus, leading to an activation of Ca^{2+}-sensitive endonuclease, fragmentation of DNA and cell death (Arnaiz *et al.*, 1995). Hydrogen peroxide and superoxide anion radical are produced during metabolic activation of paracetamol in the mixed function oxidase system (Sumioka *et al.*, 2004). Peroxynitrite is normally detoxified by GSH, which is depleted in paracetamol toxicity. NO synthesis (serum nitrate plus nitrite) was dramatically increased following acetaminophen activity .

Fig. 5 Role of oxidative stress in acetaminophen toxicity

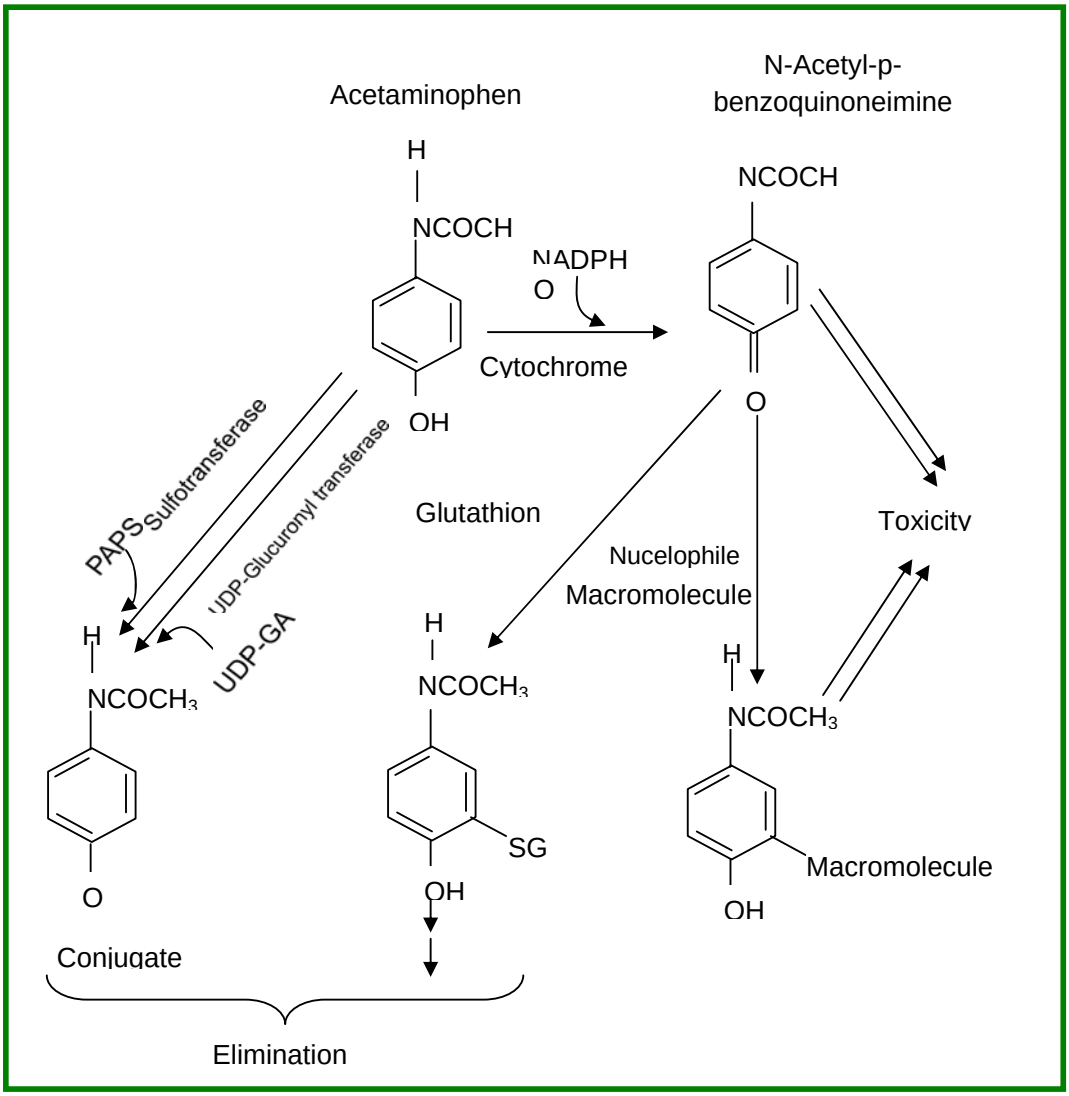


Metabolic Activation of Acetaminophen

Dr. Gillette's laboratory firmly studied the importance of metabolism in acetaminophen toxicity. Acetaminophen is metabolically activated by cytochrome P450 to form a reactive metabolite that covalently binds to protein. The reactive metabolite was found to be *N*-acetyl-*p*-benzoquinoneimine (NAPQI), which is formed by a direct two-electron oxidation. They also revealed that NAPQI is detoxified by glutathione (GSH) to form an acetaminophen-GSH conjugate. After a toxic dose of acetaminophen, total hepatic GSH is depleted by as much as 90%, and as a result, the metabolite covalently binds to cysteine groups on protein, forming acetaminophen-protein adducts. This mechanism is shown in Fig.6

Chapter 6 Results

Fig. 6 Role of metabolism in acetaminophen toxicity

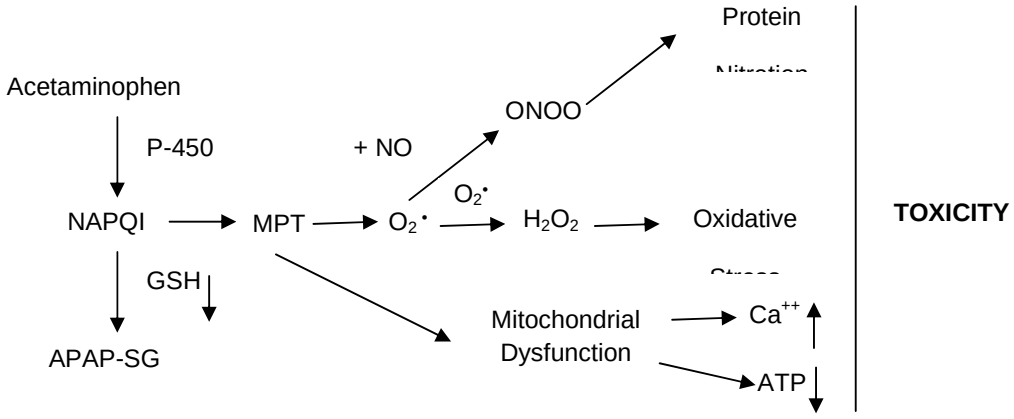


Superoxide Formation and Mitochondrial Dysfunction in Acetaminophen Toxicity

Superoxide may be formed from the CYP-450 and other enzymes. Investigation is carried out on the importance of activation of kuffer cells, macrophages, or neutrophils (the so-called respiratory burst) in acetaminophen toxicity. Finally, it was concluded that superoxide anion is released from increased activity of enzyme, NADPH-oxidase. Mitochondrial dysfunction may be formed via occurring the MPT (mitochondrial transition permeability) with formation of superoxide anion radicals which leads to peroxynitrite and tyrosine nitration. The toxicity was mediated by oxidants such as peroxides, peroxynitrite, increased calcium, Pi promote the onset of MPT, mg2+, ADP, low pH, high membrane potential, ROS and decreased ATP .

Fig. 7 Role of mitichondrial permeability transition in

acetaminophen toxicity



DIFFERENT EXPERIMENTAL MODELS USED TO EVALUATE HEPATOPROTECTIVE ACTIVITY

Both *in vitro* and *in vivo* models are available for the evaluation of hepatoprotective activity.

I) *In vitro* liver models

The most frequently used isolated liver preparations include isolated perfused organs, precision cut liver slices, sub-cellular fractions and isolated and cultured liver cells (Groneberg, 2002).

i) Isolated perfused organ

The isolated perfused organ displays an approach towards the assessment of organ physiology and morphology and represent the closest model to the *in vivo* situation. The major advantages are that the preservation of the 3-dimensional organ structure with all its cell-to-cell interactions and the possibility of real-time bile collection and analysis. This model allows the study of hemodynamic parameters if blood is used as a perfusate. The different models of isolated perfused livers proved to be very complex in keeping organ function within physiological ranges, and their functional integrity was never

maintained over a prolonged period. Also the establishment of these models is very expensive.

ii) Precision cut liver slices

The precision-cut liver slice model can be used to examine the cellular aspects of liver toxicology in a tissue-specific background. The liver slice was one of the first preparations used to study *in vitro* liver metabolism. This model helps to retain tissue organization and cell-to-cell matrix interactions such as perfused organs. The main advantages are represented by the preservation of lobular structures in contrast to cell cultures and the possible application of biochemical and molecular biological methods in contrast to organ perfusions. This system can be used for a period of 2 to 3 days as a valid model to study hepatotoxicity and even human tissues can be used after surgical or needle biopsy removal.

iii) Sub-cellular fractions

These models can be obtained from animals pretreated by inducers and are useful to study the mechanisms of hepatotoxicity. Microsomes are widely used to identify drug metabolic pathways, covalent binding and lipid peroxidation induced by hepatotoxins. Isolated mitochondria are widely used in the study of the effect of toxicants on cellular energy transformation.

iv) Liver cells

a) Isolated and cultured hepatocytes

The liver cell culture model can be applied to examine the effects of drugs/toxins on isolated hepatocytes on the cellular level. Berry and Friend established the basic protocol involving a two-step perfusion of the liver, first with calcium free buffer, followed by calcium-supplemented buffer containing collaginase. The advantages of the use of freshly isolated hepatocytes

are the homogenicity of the suspension, functional similarities with the *in vivo* state and the ability to analyze multiple parameters from a single cell suspension. To guarantee the survival of hepatocytes isolated from individual donors, cryopreservation or cold storage techniques can be applied that lead to an indefinite or 48 h extension respectively. The viability of stored cells is much lower than that of freshly isolated hepatocytes and dependent on factors such as initial cell integrity, ice crystal formation, and hypoxia during freezing and toxicity of cryopreservation substances. Hepatocytes have the capacity of biotransformation which is crucial for toxicological studies. A significant disadvantage in the hepatocyte culture is the absence of organ-specific cell-to-cell interactions.

b) Liver cell lines

Liver cell lines can be obtained from hepatomas or

transfected hepatocytes. Hepatoma cell lines are difficult to establish from primary carcinomas and exhibits only some of the characteristics of normal liver.

II) *In vivo* models

Whole animal as experimental model

The studies performed during the past 100 years have employed a variety of species, the most popular being mice because of their size and relatively low cost. Accordingly, most of the accumulated information bearing on experimental hepatotoxicity and on modifier of susceptibility, such as age, sex, stage of development, diet and exposure to other toxic substance, applies to the mice. The general employment of relatively uniform experimental model permits comparison of results obtained in widely separated laboratories. To a varying degree, rat, hamsters, guinea pigs, rabbits, dogs, cats, cattle, horse, sheep, and several species of birds have been employed, and the studies of any particular

chemical may include any of these or other species. During recent years, primates have come to use, for the obvious reason of the greater presumed relevance to the disease of humans.

PARAMETERS OF INJURY

Measure of hepatic injury includes lethality, histological changes seen by light electron microscopy, chemical changes seen in the liver and biochemical tests that measure the functional status or that reflect the type or intensity of hepatic injury.

1) Lethality

Death as a measure of hepatotoxic potency is applicable mainly to known hepatotoxins. Employment of the LD_{50} or other measures of lethal potency permits comparison of hepatotoxic agents.

2) Histology

Toxic hepatic injury can be categorized by using light

microscopy and scanning electron microscopy.

a) Light microscopy

LM is the traditional method for demonstrating toxic hepatic injury and categorizing its type. It provides yardstick against which other abnormalities can be measured. However, LM provides only a crude estimation for the quantitation of the degree of injury.

b) Electron microscopy

It provides a much earlier demonstration of hepatocyte injury and permits the recognition of damage too subtle to be appreciated by LM. It is also useful in differentiating lesions that appear to be similar in LM. For e.g. hepatic injury induced in mice by galactosamine resembles that of viral hepatitis when examined by LM, but not when examined by EM. It provides evidence earlier than LM and may yield clues to the mechanisms of injury.

c) Scanning electron microscopy

This approach to ultra structural studies appears to have added new dimensions to the study of structural changes induced by toxic agents. Studies of cholestatic effects of hepatotoxins utilizing the scanning technique provided the database for a new hypothesis for the development of cholestasis.

Enzymatic antioxidants

The term antioxidant has been defined by Halliwell and Gutteridge as any substance that delays or inhibits oxidative damage to a target molecule. The first line of defense against superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) mediated injury are antioxidants enzyme like superoxide dismutase (SOD), glutathione peroxides (GPx), and catalase (CAT).

Lipid peroxidation (LPO)

Malondialdehyde (MDA) is the major reactive aldehyde resulting from the peroxidation of biological membrane polyunsaturated fatty acids (PUFA). MDA, a secondary product of LPO, is used as an indicator of tissue damage by a series of chain reactions. MDA is also a byproduct of prostaglandin biosynthesis. It reacts with thiobarbituric acid and produces a red-coloured product. MDA is a mutagenic and genotoxic agent that may contribute to the development of human cancer (Halliwell *et al.,* 1993).

Superoxide dismutase (SOD)

SODs are a family of metalloenzymes that convert $O_2^{\cdot -}$ to H_2O_2 according to the following reaction,

$$O_2^{\cdot -} + O_2^{\cdot -} \xrightarrow{2H} H_2O_2 + O_2$$

SOD

SOD is the most important enzyme because it is found virtually in all aerobic organisms. There are four families of SOD, namely Cu-SOD, Cu-Zn-SOD, Mn-SOD, and Fe-SOD. The transition metal of the enzyme reacts with $O_{2\cdot}^{-}$ taking its electron. Superoxide anion is the only known substrate for SOD.

Cu-Zn-SOD is found in the cytosol of most eukaryotic cells. A different form of Cu-Zn-SOD is found in extra cellular fluids, where it is called EC-SOD. Mn-SOD is located in the mitochondrial matrix and bacteria, while Fe-SOD is present in many aerobic bacteria. Cu-Zn-SOD is sensitive to cyanide, but is destroyed by the treatment with chloroform plus ethanol. SOD is considered to be a stress protein, which is synthesized in response to oxidative stress.

SOD has been detected in a large number of tissue

and organisms, and is thought to protect the cell from damage caused by $O_2^{\cdot-}$ and OH radicals generated from the metal-catalysed interaction of O_2 with H_2O_2 (Halliwell *et al.*, 1993).

Glutathione (GSH)

Glutathione is a major antioxidant, critical to the protection of tissues from free radical injury. GSH is a ubiquitous tri-peptide formed from three aminoacids glutamate, glycine and cysteine and synthesized by two ATP-dependent enzymatic reactions. GSH is a major intracellular antioxidant molecule. It plays a critical role in detoxification of peroxides and electrophilic toxins as a substrate for GSH peroxidase and GSH transferase. It was shown that depletion of GSH enhances cerebral ischemic injury in rats.

Glutathione peroxidase (GPx)

Glutathione peroxidase enzyme is a well-known first

line defense against oxidative stress, which in turn requires glutathione as a co-factor. GPx catalyses the oxidation of GSH to GSSG (oxidized glutathione) at the expense of H_2O_2. By its selenium dependency, GPx can be divided in two forms, Se-dependent GPx and Se-independent GPx. The former is a tetramer of MW 84000 with very high activity towards both H_2O_2 and organic hydroperoxides. It is found in both cytosol (70%) and mitochondria (30%) of various tissues. Since selenium is an integral component of GPx, the measurement of this enzyme has been used as a functional index of selenium level. GPx activity is reduced in selenium deficiency (Halliwell *et al.*, 1993).

Catalase (CAT)

Catalase is an enzyme, which is present in most cells and catalyses the decomposition of hydrogen peroxide to water and oxygen. CAT is a heme containing protein. The mechanism of the action is,

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

CAT is found to act 10^4 times faster than peroxidase. It is localized mainly in the mitochondria and in sub-cellular respiratory organelles. CAT is present in peroxisomes (80%) and cytosol (20%). It has a molecular weight of about 2,40,000 and consists of four protein subunits, each containing a heme Fe(III)-protoporphyrin group bound to its active site. GPx and CAT were found to be important in the inactivation of many environmental mutagens (Halliwell *et al.,* 1993).

HEPATOPROTECTION

Liver diseases constitute a major health problem of worldwide populations. Liver injury is induced by various pathogenic factors such as viral hepatitis, ethanol, drugs

hepatotoxicants, etc. Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical diseases. There is a growing interest in the pharmacological evaluation of various plants used in Indian traditional system of medicine. Many research's have been directed towards the provision of empirical proof to back up the use of many plants in traditional medical practice. However, there still exist a vast number of plants with tremendous medicinal potential but with no empirical proof to support claims of efficacy (Halliwell et al., 1993).

2. PLANT PROFILE

Desmostachya bipinnata (L.)Stapf

Family : Poaceae

Vernalcular names

English : Sacrificial grass

Hindi : Davoli

Malayalam : Balidarbha

Sanskrit : Darbhah

Tamil : Darbhaibhul

Telugu :

Aswalayana,Darbha,Kushadarbha.

Habitat

Perennial grass, found throughout India in hot and dry places.

Description

Perennial grass, tall, branched from the base, root stock stout, creeping, stolons very stout, covered with shining sheathes, stems 30-90cms high, tufted, smooth, erect, stout. leaves many, the basal fascicled, reaching sometimes 50cm long and 1cm broad at the base, rigid, acuminate, with filiform tips and hispid margins, sheath glabrous, ligule a hairy line. Panicles erect, narrowly pyramidal clothed from the base with sessile imbricating spikelets, grain 0.5-0.6mm long, obliquely ovoid, laterally compressed.

Parts used

Whole plant is used.

Constituents

Flavonoids, glycosides, saponins, tannins, carbohydrate .

Properties

Diuretic, antidermatic, Aphrodisiac, galatogogue.

Medicinal uses

It is useful in thirst, asthma. Jaundice, disease of the blood, disease of the bladder, skin eruption, dysentery, uropathy, strangury, vesical calculi. (Krithikar and Basu, 1987).

Fig. 8 *Desmostachya bipinnata* (L.) Stapf.

3. REVIEW OF LITERATURE

Akare *et al*., (2009) reported that the ethanol and aqueous extracts of *Acacia ferruginea* leaves were tested for their efficacy against carbon tetrachloride (CCL_4) induced hepatotoxicity in Wistar albino rats. The different groups of animals were administered with CCL_4 (1ml/kg, s.c.). The ethanol and aqueous extracts at the dose of 200mg/kg were administered to CCL_4 treated rats.The result of present study demonstrated that ethanol extract significantly decreases the level of alanine aminotransferase, aspartase aminotransferase, total bilirubin and direct bilirubin in blood, as compare to aqueous extract. The phytochemical screening revealed the presence of active phytoconstituents i.e. flavonoids and tannins, which may offer

hepatoprotection. The present work support the traditional claim of plant in the treatment of liver injury, may provide a new drug against a war with liver diseases.

Satyanarayana *et al*., (2009) evaluated the hepatoprotective effect of the alcohol extract of *Capparis sepiaria* Linn. (Capparaceae) stem against carbon tetrachloride (CCl_4)-induced toxicity in albino rats. The rats were given daily pretreatment with alcohol extract of *C. sepiaria* (100 mg/kg) and the standard silymarin (25 mg/kg) orally for 7 days. The toxicant used on 7th day was CCl_4 at a dose of 1.25 ml/kg as 1:1 mixture with olive oil. The extract produced significant ($p<0.01$) reduction in the elevated levels of aspartate transaminase (AST), alanine transaminase (ALT), total bilirubin (TB) and rise of decreased total protein level when compared with the toxic control.

Vadivu *et al*., (2009) reported the hepatoprotective and *in-vitro* cytotoxic activity of alcoholic extract of leaves of *Premna serratifolia*

Linn. Hepatoprotective activity was studied by carbon tetrachloride - induced hepatotoxicity in rats and the *in-vitro* cytotoxic activity is carried out by tryphane blue exclusion method using EAC cell lines. The degree of protection in hepatoprotective activity has been measured by using biochemical parameters such as serum glutamate oxalate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), bilirubin and total protein. The results suggest that the alcoholic extract at the dose level of 250mg/kg has produced significant (p<0.001) hepatoprotection by decreasing the activity of serum enzymes, bilirubin, and lipid peroxidation which is comparable to that of standard drug silymarin. The alcoholic extract also does exhibit the IC_{50} value of 75µg/ml which indicates the significant *in-vitro* cytotoxic activity of the extract**.** It is concluded that alcoholic extract of leaves of *Premna serratifolia* Linn is not only an effective hepatoprotective agent, but also possesses significant antitumor activity.

Awaad *et al*., (2008) isolated the five main flavonoid glycosides from the ethanol extract of *Desmostachia bipinnata* (L.)Stapf (Gramineae). They were identified as

kaempferol(1), quercetin(2), quercetin-3-glucoside(3), trycin(4) and trycin-7-glucoside(5). The structure elucidation was based on UV, Electrospray ionization mass spectrometry (ESIMS), 1H and 13C NMR, proton- proton correlation spectroscopy (1H-1H Cosy), distortionless enhancement by polarization transfer (DEPT), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlations spectrum (HMBC). The total extract (200 and 300 mg/kg) and two of the isolated compounds (trycin and trycin-7-glucoside.100 mg/kg each) showed a very promising antiulcerogenic activity.

Donfack *et al*., (2008) subjected the 40% hydroethanolic stem bark extract (HE40) from *Erythrina senegalensis* was subjected to purification by repeated column chromatography. Three diprenylated isoflavonoids were isolated and identified as 2, 3-dihydro-2'-hydroxyosajin (1), osajin (2) and 6, 8-diprenylgenistein

(3). These compounds were tested for hepatoprotective activities against *in vitro* CCl4-induced hepatitis in rat liver slices. The following four model systems were used to measure the antioxidant activity of these three isoflavones: 2,4-dinitrophenyl-1-picrylhydrazyl (DPPH) radical scavenging activities, β- Carotene-Linoleic Acid Model System (β-CLAMS), Ferric-Reducing Antioxidant Power (FRAP) assay and microsomal lipid peroxidation. By comparison to compound (2) and (3), compound (1) showed significant antioxidant effect with EC50 values of 41.28±1.2, 31.27± 2.14, 19.17±1.2 and 15.99±0.49 µg/ml respectively for the radical-scavenging action, inhibition of microsomal lipid peroxidation, β-CLAMS and FRAP assays. The hepatoprotective activity of silymarin used as reference compound was lower than the activities of isolated compounds. The results obtained provide promising baseline information for the potential use of this crude extract as well as some of the isolated compounds for their hepatoprotective and antioxidant activities. It is

also worth noting that these results validate, by *in vitro* tests, the therapeutic use of the plant in traditional medicine.

Mohammed *et al.*, (2008) reported the hepatoprotective effect of alcoholic and water extract of *Annona squamosa* (custard apple). These extracts were used to study the Hepatoprotective effect in isoniazid + rifampicin induced hepatotoxic model. There was a significant decrease in total bilirubin accompanied by significant increase in the level of total protein and also significant decrease in ALP, AST, ALT and γ-GT in treatment group as compared to the hepatotoxic group. In the histopathological study the hepatotoxic group showed hepatocytic necrosis and inflammation in the centrilobular region with portal triaditis. The treatment group showed minimal inflammation with moderate portal triaditis and their lobular architecture was normal. It should be concluded that the extracts of *Annona squamosa* were

not able to revert completely hepatic injury induced by isoniazid + rifampicin, but it could limit the effect of these drugs in liver. The effect of extracts compared with standard drug silymarin.

Manokaran *et al.*, (2008) evaluated the hepatoprotective activity of hydroalcoholic extract of *Aerva lanata* against paracetamol induced liver damage in rats. The hydroalcoholic extract of *Aerva lanata* (600mg/kg) was administered orally to the animals with hepatotoxicity induced by paracetamol (3gm/kg). Silymarin (25mg/kg) was given as reference standard. All the test drugs were administered orally by suspending in 0.5% Carboxy methyl cellulose solution. The plant extract was effective in protecting the liver against the injury induced by paracetamol in rats. This was evident from significant reduction in serum enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and bilirubin. It was concluded

from the result that the hydroalcoholic extract of *Aerva lanata* possesses hepatoprotective activity against paracetamol induced hepatotoxicity in rats.

Shi *et al.*, (2008) reported the hepatoprotective effect of *Ganoderma lucidum* peptides (GLP) against D-galactosamine (D-GalN)-induced liver injury in mice. *Ganoderma lucidum* is a traditional Chinese medicinal mushroom. GLP was administered orally for 2 weeks daily at doses of 60, 120 and 180 mg/kg. Control groups were given same amount of saline. After 2 weeks GLP-treated groups were treated with D-GalN (750 mg/kg) suspended in normal saline by intraperitoneal injection. D-GalN induced hepatic injury was manifested by a significant increase in the activities of marker enzymes (AST, ALT) in serum and MDA levels in liver and by a significant decrease in activity of SOD and GSH level in liver. Pre treatment of mice with GLP reversed these altered parameters to normal value. The best hepatoprotective

effects of GLP were observed after treatment with the dose of 180 mg/kg.

Asha *et al.*, (2007) evaluated the hepatoprotecttive activity of *Phyllanthus maderaspatensis* against experimentally induced liver injury in rats. In this study the hexane extract of *Phyllanthus maderaspatensis* (200 and 100 mg/kg) showed significant hepatoprotection on carbon tetrachloride and thioacetamide induced liver damage in rats. The protective effect was evident from serum biochemical parameters and histopathological analysis. Rats treated with *P.maderaspatensis* remarkably prevented the elevation of serum AST, ALT and LDH and liver lipid peroxides in carbon tetrachloride and thioacetamide treated rats. Hepatic glutathione levels significantly increased by the treatment with the extracts. The activity of the extract was comparable to that of silymarin, the reference hepatoprotective drug.

Pramyothin *et al.*, (2007) reported the *in vitro* and *in vivo* hepatoprotective activity of *Phyllanthus amarus*

Schum extract in ethanol treated animals. In the *in vitro* study, *Phyllanthus amarus* (PA) (1-4 mg/ml) increased % MTT reduction assay and decreased the release of transaminases (ALT and AST) in rat primary cultured hepatocytes being treated with ethanol. Treatment of rats with PA (75 mg/kg day, p.o) or silymarin (5 mg/kg, p.o) for 7 days after 21 days with ethanol (4 g/kg day,p.o) enhanced liver cell recovery by bringing the levels of AST, ALT, HTG back to normal. Histopathological studies also confirmed the beneficial role of PA.

Setty *et al.*, (2007) evaluated hepatoprotective activity of *Calotropis procera* flowers against paracetamol-induced hepatic injury in rats. In their study hydroethanolic extract of *Calotropis procera* was prepared and tested for its hepatoprotective effect. Alteration in the levels of biochemical markers of hepatic damage like SGPT, SGOT, ALP, HDL, bilirubin, cholesterol and tissue GSH were tested in both treated and untreated

groups. Paracetamol (2 g/kg) has enhanced the SGPT, SGOT, ALP, bilirubin and cholesterol levels and reduced the serum levels of HDL and tissue level of GSH. Treatment with hydroethanollic extract of *C.procera* flowers (200 mg/kg and 400 mg/kg) has brought back the altered levels of biochemical markers to the normal in the dose dependent manner.

Gupta *et al.,* (2006) reported the hepatoprotective activity of aqueous ethanolic extract of *Chamomile recutita capitula* in paracetamol intoxicated albino rats. In this study the effect of aqueous ethanolic extract of *Chamomile recutita capitula* on blood and liver glutathione, Na^+ K^+ -ATPase activity, serum marker enzymes, serum bilirubin, glycogen and thiobarbituric acid reactive substances against paracetamol induced damage in rats have been studied to find out the possible mechanism of hepatoprotection. Albino rats were administered with paracetamol at a dose of 200 mg/kg

p.o and *capitula* extract at a fixed dose of 400 mg/kg p.o. The results suggested that the hepatoprotective activity of *chamomile* may be due to normalization of impaired membrane function activity.

Kumar *et al.*, (2006) reported the protective effect of root extract of *Operculina turpenthum* Linn against paracetamol-induced hepatotoxicity in rats. The ethanolic extract obtained from roots of *Operculina turpenthum* were evaluated for hepatoprotective activity in rats by inducing liver damage by paracetamol. The ethanol extract at an oral dose of 200 mg/kg exhibited a significant protective effect by lowering serum levels of glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, alkaline phosphatase, and total bilirubin. These biochemical observations were supplemented by histopathological examination of liver sections. Hepatotoxicity was developed by inducing paracetamol at a dose of 3 g/kg orally. Silymarin was used

as the reference drug and was administered at a dose of 200 mg/kg orally.

Manjunatha *et al*., (2006) evaluated the hepatoprotective activity of crude aqueous and ethanol bark extracts of *Pterocarpus santalinus* (Fabaceae) using CCl_4 induced hepatic damage in male Wistar rats. The hepatoprotective activity was assessed using various biochemical parameters like serum albumin, protein, alanine transaminase and alkaline phosphatase along with histopathological studies of liver tissue. There was significant increase in serum levels of billurubin, alanine transaminase and alkaline phosphatase with a decrease in total protein levels in CCl_4 treated animals. The ethanol and aqueous stem bark extract of *P.santalinus* afforded significant protection against CCl_4 induced hepatocellular injury.

Yen *et al.*, (2006) reported the hepatoprotective and antioxidant effects of *Cuscuta chinensis* against acetaminophen-induced hepatotoxicity in rats. They evaluated and compared the hepatoprotective and antioxidant activities of the aqueous and ethanolic extract of *C.chinensis* on APAP induced hepatotoxicity in rats. The ethanolic extract of *C chinensis* at an oral dose of 125 and 250 mg/kg showed a significant hepatoprotective activity by reducing levels of glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), and alkaline phosphatase (ALP) and it also showed a significant antioxidant activity by increasing levels of super oxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) and by reducing malondialdehyde (MDA) levels. The same dose of aqueous extract did not showed any hepatoprotective effect as seen in ethanolic extract. The results suggest that the ethanolic extract of *C.chinensis* can prevent hepatic

injuries from APAP-induced hepatotoxicity in rats and this likely mediated through its antioxidant activities.

Murugesh *et al.*, (2005) evaluated the hepatoprotective and antioxidant role of *Berberis tinctoria* Lesch leaves on paracetamol - induced hepatic damage in rats. In this method the methanol extract of *Berberis tinctoria* Lesch leaves (MEBT) was investigated for its hepatoprotective and antioxidant effects on paracetamol (750 mg/kg) induced acute liver damage in Wistar albino rats. The MEBT at the doses of 150 mg/kg and 300 mg/kg produced significant hepatoprotective effect by decreasing the activity of serum enzymes, bilirubin, and lipid peroxidation, while it significantly increased the levels of glutathione (GSH), catalase (CAT), and super oxide dismutase (SOD) in a dose dependent manner.

Pramyothin *et al.*, (2005) evaluated the *in vitro* and *in vivo* hepatoprotective activities of *Thunbergia laurifolia* Linn extract, in rats treated with ethanol. In this study the

primary cultures of rat hepatocyte and rats were used as the *in vitro* and *in vivo* models to evaluate the hepatoprotective activity of aqueous extract from *Thunbergia laurifolia* (TLE). Ethanol was used as the hepatotoxin and silymarin as the reference hepatoprotective agent. In the *in vitro* study MTT reduction assay and the release of transaminases (ALT and AST) were the criteria for cell viability. Primary cultures of rat hepatocyte (24 h culturing) were treated with ethanol (96 µl/ml) and various concentrations of TLE or silymarin for 2 h. Ethanol decreased MTT (%) nearly by half. Both TLE and SL increased MTT reduction and brought MTT (%) back to normal. Ethanol induced release of ALT and AST was also reduced by TLE (2.5 and 5.0 mg/ml) and SL (1 mg/ml). In the *in vivo* study TLE at 25 mg/kg day po and silymarin 5 mg/kg day po, for 7 days after ethanol enhanced liver cell recovery by bringing HTg, ALT and AST back to normal. The results suggested that TLE and SL possess the hepatoprotective activity against ethanol

induced liver injury in both primary cultures of rat hepatocyte and rats.

Umamaheswari and Rao, (2005) assesed the hepatoprotective effect of Grape seed oil (GSO) against paracetamol. The hepatoprotective activity was evaluated on the basis of biochemical and histopathological studies. The serum enzyme levels (AST, ALT and ALP) were estimated by standard biochemical procedures using an auto analyzer. Grape seed oil reversed the biochemical and histopathological changes in the liver induced by paracetamol. GSO was shown to cause an increase in glutathione and total protein levels and a decrease in lipid peroxidation in paracetamol induced hepatic damage in rats. The above studies suggest that Grape seed oil offers vast possibilities in the treatment of various liver disorders. This may be due to the high level of anti-oxidant vitamin E, which was claimed to be the mechanism of hepatoprotection.

Gupta *et al.*, (2004) reported the antioxidant and hepatoprotective effects of *Bauhinia racemosa* against paracetamol and carbon tetrachloride - induced liver damage in rats. In this study different groups of animals were administered with paracetamol {500 mg/kg p.o} once in a day for 7 days and carbon tetrachloride {30 % (1 ml/kg b.wt). in liquid paraffin 3 doses (i.p) at 72 h interval}. The methanolic extract of *Bauhinia racemosa* (MEBR) was administered at the doses of 50, 100, and 200 mg/kg and silymarin 25 mg/kg were administered to the animals. The MEBR and silymarin produced significant hepatoprotective effect by decreasing the activity of serum enzymes, bilirubin, and lipid peroxidation and increased the levels of GSH, SOD, CAT, and protein in a dose dependent manner. MEBR also showed antioxidant effects on $FeCl_2$-ascorbate-induced lipid peroxidation in rat liver homogenate and on superoxide scavenging activity.

Mankani *et al*., (2004) reported the hepatoprotective activity of *Pterocarpus marsupium* stem bark extracts against carbon tetrachloride (CCl_4)-induced hepatotoxicity. Hepatotoxicity was induced in male *wistar* rats by intraperitoneal injection of CCl_4 (0.1 ml/kg/day for 10 days). Methanol and aqueous extracts of P. marsupium stem bark were administered to the experimental rats (25 mg/kg/day, p.o. for 14 days). The hepatoprotective effect of these extracts was evaluated by the assay of liver function biochemical parameters (total bilirubin, serum protein, alanine aminotransaminase, aspartate aminotransaminase, and alkaline phosphatase activities) and histopathological studies of the liver. In methanol extract-treated animals, the toxic effect of CCl_4 was controlled significantly by restoration of the levels of serum bilirubin, protein and enzymes as compared to the normal and the standard drug silymarin-treated groups. Histology of the liver sections of the animals treated with the

extracts showed the presence of normal hepatic cords, absence

of necrosis and fatty infiltration, which further evidenced the hepatoprotective activity.

Naik *et al.*, (2004) reported the protection of liver cells from ethanol cytotoxicity by curcumin in liver slice culture *in vitro*. In this study liver slice culture model was used to demonstrate hepatoprotective activity of curcumin *in vitro*. Ethanol was used as the hepatotoxin and the cytotoxicity of ethanol was estimated by quantitating the release of LDH. Ethanol induces 3.5 times more release of LDH from the liver cells and twice the amount of lipid peroxidation as compared to the cells from untreated liver tissue and this was significantly reduced in presence of curcumin (5 μM). The activity of antioxidant enzymes like superoxide dismutase, catalase, and peroxidase were measured and found that in ethanol treated cells the activity of all three enzymes was

elevated. But when curcumin was added along with ethanol their levels were kept low.

Pandian *et al.*, (2004) reported the hepatoprotective activity of *Trianthema portulacastrum* L. against paracetamol and thioacetamide intoxication in albino rats. The ethanolic extract of *Trianthema portulacastrum* L. showed a significant dose dependent (100 mg, 200 mg/kg p.o) protective effect. Paracetamol was administered at a dose of 3 g/kg p.o and thioacetamide induced hepatotoxicity was done by injecting thioacetamide (100 mg/kg s.c) as a 2 % w/v solution in distilled water. The degree of protection was measured by using biochemical parameters like serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), bilirubin and total protein (TP). The plant extract completely prevented the toxic effects of paracetamol and thioacetamide on the above serum parameters.

Raza *et al.*, (2003) performed a comparison of hepatoprotective activities of aminoguanidine (AG) and N-acetylcysteine (NAC) in rat against the toxic damage induced by azathioprine (AZA). The rationale behind this study was the proven efficacy of N-acetylcysteine, and reports on the antioxidant potential of aminoguanidine that might be useful to protect against the toxic implications of AZA. AG (100 mg/kg i.p) or NAC (100 mg/kg i.p) were administered to the Wistar male rats for 7 days and after that AZA (15 mg/kg i.p) was given as a single dose. This caused an increase in the activity of hepatic aminotransferases (AST and ALT) in the serum 24 h after AZA treatment and also caused an increase in rat liver lipid peroxides and a lowering of reduced glutathione (GSH). Pretreatment with NAC prevented any change in the activities of both the amino transferases after AZA. This also resulted in a significant decline in the contents of lipid peroxides and a significant elevation in GSH level was evident after AZA treatment. In the group with AG

pretreatment the activities of AST and ALT did not increase significantly after AZA when compared to control. However the lipid peroxides and GSH levels did not have any significant difference when compare to AZA group. These indicate that the improvement in the GSH levels by NAC is the most significant protective mechanism. The protective effect of AG against the enzyme leakage seems to be through the liver cell membrane permeability restoration and is independent of any effects on liver GSH contents.

Czinner *et al.*, (2001) evaluated the *in vitro* effect of *Helichrysi flos* on microsomal lipid peroxidation. The aim of this study was to verify the antioxidant properties of lyophilized water extracts with different polyphenol and flavanoid contents from inflorescences. The effects of natural extracts on microsomal fraction of rat liver were examined. Enzymatically induced lipid peroxidation and NADPH cytochrome *c* reductase activity in liver

microsomes were measured by spectrophotometric methods. Results were compared with the activity of silibinin flavanoid , the main agent of well-known milk thistle. The natural plant extracts diminish the enzymatically induced lipid peroxidation in a concentration-dependent manner and reduce the cytochrome *c* dose dependently. The lyophilized *Helichrysi flos* extracts proved to be more effective compared to silibinin in examined concentrations.

Reen *et al.*, (2001) reported the screening of various *Swertia* species extracts in primary monolayer cultures of rat hepatocytes against carbon tetrachloride and paracetamol-induced toxicity. In this study eight species of *Swertia* were collected and was extracted into methanol, the aqueous extract of which was sequentially extracted into hexane, chloroform and butanol extracts. The extracts were screened for their anti-hepatotoxic effect against carbon tetrachloride and paracetamol

(AAP) toxicity in primary monolayer cultures of rat hepatocytes. The primary cultures, 2.5×10^6 cells/ 3 ml medium/ 60 mm collagen coated plates, were exposed to 2.5 mM CCl_4 or 12 mM paracetamol in the presence or absence of plant extracts (100 µg/ml). Cells and medium were harvested after 22 h of treatment for the assay of cellular reduced glutathione (GSH) content and leakage of lactate dehydrogenase as biological end points of toxicity. Both CCl_4 and AAP at the indicated concentrations reduced GSH by almost 50 and 80%, while the enzyme leakage was almost 15 % above the untreated control. Hexane and methanol extracts offered relatively good protection.

4 OBJECTIVE & PLAN OF WORK

Liver is the largest gland in the body and is an extremely active organ. Many drugs undergo chemical changes in the liver before excretion in bile or by other organs. They may damage the liver cells in their original form or while in various intermediate stages. Paracetamol (also known as Acetaminophen) is a widely used analgesic and antipyretic drug available as an over the counter medication. It is generally harmless at therapeutic doses, but large doses of acetaminophen causes acute centrilobular hepatic necrosis in mice, rats and man. Paracetamol toxicity in hepatocytes initiates a sequence of events that eventually leads to cell death. Toxic doses of paracetamol will deplete the levels of hepatic reduced

glutathione (GSH) followed by covalent binding of the reactive metabolite N-acetyl–p–benzoquinoneimine (NAPQI) formed during the biotransformation reaction by microsomal cytochrome P450 mixed function oxidase to tissue proteins. When GSH levels are low, the reactive metabolite fails to be detoxified by conjugation leading to the accumulation of NAPQI, which plays a major role in hepatic oxidative stress. Antioxidants can inhibit these events suggesting that deleterious oxidative changes are involved.

In recent years, many researchers have examined the effect of plants used traditionally by indigenous people to support liver function and plants used traditionally by indigenous people to support liver function and treat disease of the liver. In most cases, research has confirmed traditional experience by discovering the mechanism and mode of action of these plants. *Desmostachya bipinnata* (L.) Stapf belonging to the family

Poaceae is a grass grown abundantly in various parts of India. The plant contains carbohydrates, flavonoids, glycosides, saponins and tannins as constituents. The different parts of the plant are used to treat ulcers, skin eruption, dysentery, liver disorders, asthma.

The objective of the present study is to evaluate the hepatoprotective activity of the leaves of *Desmostachya bipinnata* (L.) Stapf against paracetamol-induced hepatotoxicity using *in vitro* models.

PLAN OF WORK

The work involved the following steps,

- Collection and authentification of the leaves of *Desmostachya bipinnata* (L.) Stapf.

- Preparation of ethanolic extract and fractions.
- Phytochemical screening.
- *In vitro* studies using liver slice culture method.
- Estimation of lactate dehydrogenase.
- Estimation of tissue protein, malondialdehyde and lipid hydroperoxides in experimental animals.
- Estimation of liver enzymatic antioxidants like superoxide dismutase, catalase, glutathione peroxidase, peroxidase, glutathione reductase in experimental animals.
- Estimation of liver non-enzymatic antioxidant like reduced glutathione.
- Histopathological studies of the liver.
- Statistical analysis.

5 MATERIALS AND METHODS

Plant Material

The plant material consists of dried powdered leaves of *Desmostachya bipinnata* (L.) Stapf belonging to the family Poaceae. Aerial parts of the plant sample was air-dried in shade, reduced to fine powder, packed in tightly closed container and stored for phytochemical and pharmacological studies.

Plant collection and authentication

The aerial part of *Desmostachya bipinnata* (L.) Stapf were collected from Alapuzha district, Kerala during the month of July 2009. The plant was identified and authenticated by Dr. G.V.S. Murthy, Joint Director, Botanical Survey of India, Tamil Nadu Agricultural University Campus, Coimbatore bearing the reference number BSI. /SC/5/23/09-10 Tech-436.

Preparation of the extract and fractionation

Dried aerial parts of *Desmostachya bipinnata* (L.) Stapf (200g) were extracted in a soxhlet apparatus with 95% ethanol (1800ml).The ethanol extract was completely dried under vacuum and weighed. The percentage yield was found to be 15.02%w/w. Then the ethanolic extract of the leaves of *Desmostachya bipinnata* (L.) Stapf was partitioned against petroleum ether, n-butanol, chloroform and ethyl acetate separately in the order of

increasing polarity. Then the extract was mixed with organic solvent (50ml) in a separating funnel and shaken for 30 minutes. The organic layer was separated and fresh solvent was added until the recovery of clear organic layer. Then, the solvents were completely removed from the individual fractions under reduced pressure to obtain the petroleum ether fraction (PEF), n-Butanol fraction (nBF), chloroform fraction (CF), ethyl acetate fraction (EAF). They were subsequently subjected to preliminary phytochemical analysis and pharmacological studies.

Drugs and chemicals

Paracetamol was obtained as a gift sample from Eurochem laboratories Ltd, Alathur, Tamil Nadu. Silymarin was obtained from Microlabs, Bangalore. Thiobarbituric acid, trichloro acetic acid, butylated hydroxyl toluene, oxidized glutathione, epinephrine and 2,2 dithiobis 2-nitrobenzoic acid (DTNB) were obtained from Sisco Research Laboratries Pvt. Ltd., Mumbai. 2,2 dipyridyl and O-dianisidine were obtained from Himedia Laboratries

Ltd., Mumbai. All other drugs and chemicals used in the study were obtained commercially and were of analytical grade.

Materials & Equipments required

- Surgical-blades
- Soxlet apparatus
- Separating funnel
- 20 ml capped glass beakers
- Tissue homogenizer
- Centrifuge
- Incubator
- Spectrophotometer
- Glass wares

Phytochemical screening

Chemical tests were carried out for the various fractions of *Desmostachya bipinnata* for the presence of phytochemical constituents (Trease and Evans, 2002).

Test for tannins and phenolics

To the solution of each fraction, a few drops of 0.1%

ferric chloride was added and observed for brownish green or a bluish-black colouration.

Test for Glycosides

A portion of each fraction was mixed with few drops of Fehling's solution A and B and heated gently. A brick red precipitate indicates the presence of glycosides.

Test for saponins

About 10 ml of each fraction was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent-froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously and then observed for the formation of an emulsion.

Test for flavonoids

1. To a portion of the fraction, concentrated H_2SO_4 was added. A yellow coloration indicates the presence of flavanoids. The yellow colouration disappeared on standing.
2. Few drops of 1% $AlCl_3$ solutions was added to a

portion of each fraction. A yellow coloration indicates the presence of flavonoids.

3. A portion of the fraction was heated with 10 ml of ethylacetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow coloration indicates a positive test for flavonoids.

Test for carbohydrates

A small quantity of the fractions was dissolved separately in 4 ml of distilled water and filtered. The filtrate was subjected to the following testes to detect the presence of carbohydrate and glycosides.

(a) Molisch's test

The filtrate was treated with 2-3 drops of 1% alcoholic a-napthol solution and 2 ml of concentrated H_2SO_4 was added along the sides of the test tube.

Appearance of brown ring at the junction of two liquids shows the presence of carbohydrates.

(b) Fehling's test

The filtrate was treated with 1 ml of Fehling's solution A and B and heated on the water bath. A reddish precipitate was obtained shows the presence of carbohydrate.

Test for terpenoids

About 5 ml of each fraction was treated with 2 ml of chloroform and 3 ml concentrated H_2SO_4 was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids.

Test for alkaloids

A small portion of the fraction was stirred with few drops of diluted HCl and filtered.

1. To the filtrate, Dragendorff's reagent (potassium bismuth iodide solution) was added and an orange brown precipitate indicates the presence of alkaloids.

2. To the filtrate, Mayer's reagent was added and a cream precipitate indicates the presence of alkaloids.

***IN VITRO* HEPATOPROTECTIVE ACTIVITY**

Composition of Kreb's Ringer HEPES medium (KRH)

- 2.5 mM HEPES PH 7.4
- 118 mM Sodium chloride
- 2.85 mM Potassium chloride
- 2.5 mM Cacium chloride
- 1.15 mM Potassium dihydrogen phosphate
- 1.18 mM Magnesium sulphate
- 4.0 mM Glucose
- Double distilled water

Liver slice culture *in vitro*

Liver slice culture was maintained following the protocol developed by Wormser *et al.,* (1990). *Wistar* albino rats weighing about 150-200 g was dissected after cervical dislocation and liver lobes were removed and transferred to pre-warmed KRH medium. Liver was then cut into thin slices using sharp scalpel blades. The liver

slices were weighed and those weighing between 20 and 25 mg was used for the experiment. Each experimental system contained 20–22 slices weighing about 500–620 mg. The slices were washed with 10 ml KRH medium, every 10 min for a period of 1 h. They were then pre-incubated for 60 min in small plugged beakers containing 2 ml KRH medium on a shaker water bath at 37°C. At the end of the pre-incubation, the medium was replaced by fresh 2 ml KRH and incubated for 2 h at 37°C with either paracetamol or plant fractions or both. Two different experimental conditions were used for treatment with plant fractions, ie 1) Plant fractions was present for 0.5 h during pre-incubation and also for next 2h along with paracetamol. 2) Plant was present only for 2 h along with paracetamol. A portion of liver tissue in each group after respective drug treatment was preserved in 10% formalin for histopathological studies. At the end of incubation, each group of slices was homogenized in appropriate volume of chilled potassium phosphate buffer (100 mM,

pH 7.8) in an ice bath to give a tissue concentration of 100 mg/ml. The homogenates were centrifuged at 10,000 rpm for 10 min and the supernatents assayed for lactate dehydrogenase (LDH).

The experiment was divided into 5 groups as mentioned below:

Group I - Control (KRH medium alone)

Group II - Paracetamol (15 mM) alone

Group III - VI - Plant fractions (PEF, nBF, CF and EAF respectively) (100 µg/ml) was present for 0.5 h during pre-incubation and also for next 2 h with paracetamol.

Group VII - X - Plant fractions (PEF, nBF, CF and EAF respectively) was present only for 2 h along with paracetamol.

Group XI - Silymarin (100 µg/ml) was added along with paracetamol.

The supernatant was used for the assay of marker enzymes viz., aspartate amino transferase (AST), alanine amino transferase (ALT),

alkaline phosphate (ALP), and bilirubin. The enzyme levels was assayed using commercial kits obtained from Agappe diagnostics Ltd, Kerala. The results were expressed as units / liter (U/L).

SERUM BIOCHEMICAL PARAMETERS SERUM GLUTAMATE PYRUVATE TRANSAMINASE (SGPT/ALT)

Principle

$$\text{L-alanine} + \alpha\text{-ketoglutarate} \xrightarrow{\text{GPT}} \text{Pyruvate-L-glutamate}$$

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightarrow{\text{LDH}} \text{L-Lactate} + NAD^{+}$$

GPT : Glutamate Pyruvate transaminase

LDH: Lactate Dehydrogenase

Procedure

To the sample tube, 100 µl of serum and 1000 µl of working reagent were added. Mixed, incubated at 37°C and the absorbance were read at 1 min. The change in optical density per min. (ΔOD/min) at 340 nm during 3

minutes.

Calculation

SGPT activity in U/L = ΔOD/min × 1768

SERUM GLUTAMATE OXALOACETATE TRANSAMINASE (SGOT/AST)

Principle

L-aspartate + α-ketoglutarate $\xrightarrow[\text{MDH}]{\text{GOT}}$ Oxaloacetate + L-glutamate

Oxaloacetate + NADH + H^+ → Malate + NAD^+

GOT : Glutamate-oxaloacetate transaminase

MDH: Malate dehydrogenase

Procedure

To the sample tube, 100 µl of serum and 1000 µl of working reagent were added. Mixed, incubated at 37°C and the absorbance were read at 1 min. The change in optical density per min. (ΔOD/min) at 340 nm during 3

minutes.

Calculation

SGOT activity in U/L = ΔOD/min × 1768

ALKALINE PHOSPHATASE (ALP)

Principle

$$PNPP + H_2O \xrightarrow{ALP} \text{P-Nitro phenol} + \text{Phosphate}$$

PNPP : Para-nitro phenyl phosphate

ALP : Alkaline phosphatase

Procedure

To the sample tube, 20 µl of serum and 1000 µl of working reagent were added. Mixed, incubated at 37°C and the absorbance were read at1 min. The change in optical density per min. (ΔOD/min) at 405 nm during 3 minutes.

Calculation

ALP activity in U/L = ΔOD/min ×2750

TOTAL BILIRUBIN

Principle

Sulfanilic acid reacts with sodium nitrite to form in the presence of diazotized sulfanilic acid to form azobilirubin. In the absence of diethyl sulfoxide, only the direct bilirubin reacts to give azobilirubin.

Procedure

To the sample tube, 50 µl of serum, 1000 µl of total bilirubin reagent and 20µl of total activator were added. Mixed, incubated at 37°C and the absorbance were read at 1 min. The change in optical density per min. (ΔOD/min) at 546 nm during 5 minutes.

Calculation

OD of sample test-OD of sample blank

$$\text{Total bilirubin} = \frac{\text{OD of Std}}{\text{OD of Std}} \times 10$$

Estimation of protein content

Protein content of the tissue homogenate was assayed by the method of Lowry *et al.*, (1951). The blue copper developed by the reduction of the phosphomolybtic phospotungestic components in the Folin-Ciocolteau reagent by the aminoacid tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured by the Lowry's method. About 50 mg of bovine serum albumin was weighed accurately and dissolved in distilled water and made up to 50 ml in a standard flask. About 10 ml of the stock solution was diluted to 50 ml with distilled water in a standard flask. One ml of this solution contains 200 µg protein. About 0.2 to 1.0 ml of the working standard was

pipette out into series of test tube. About 0.1 ml of supernatant was pipetted out in other test tubes. The volume was made up to 1 ml in all the test tubes with distilled water. A tube with 1 ml of water served as the blank. Five ml of alkaline copper solution was added to the test tubes and allowed to stand for 5 min. Then 0.5 ml of Folin reagent was added and incubated at room temperature in dark for 30 min. The absorbance was measured at 660 nm. Protein content was expressed as µg/mg of protein (Lowry *et al.*,1951).

Estimation of malondialdehyde

Lipid peroxidation as evidenced by the formation of thiobarbituric acid reactive species (TBARS) and lipid hydroperoxides (LH) were measured by the method of Niehaus and Samuelsson (1986). About 0.1 ml of tissue homogenate (Tris HCl buffer,pH7.4)was treated with 2 ml of (1:1:1 ratio) TBA-TCA-HCl reagent (Thiobarbituric acid

0.37%, 0.25N HCl and 15% TCA) and placed in a water bath for 15 min, cooled and centrifuged at room temperature for 10 min at 1000 rpm. The absorbance of the clear supernatant was measured against a reference blank at 535 nm. The values are expressed as µmoles /min/mg protein.

Estimation of lipid hydroperoxides

About 0.1 ml of tissue homogenate was treated with 0.9 ml of Fox reagent (88 mg butylated hydroxytoluene (BHT), 7.6 mg xylenol orange and 9.8 mg ammonium ion sulphate were added to 90 ml of methanol and 10 ml 250 mM sulphuric acid) and incubated for 30 min. The colour developed was read at 560 nm using a colorimeter. The values are expressed as µ mol/mg tissue protein (Niehaus and Samuelsson, 1986).

DETERMINATION OF ENZYMATIC ANTIOXIDANTS

Estimation of superoxide dismutase (SOD)

SOD activity was determined by the inhibition of auto catalyzed adrenochrome formation in the presence of homogenate at 480 nm. The reaction mixture contained 150 µl of homogenate, 1.8 ml of carbonate buffer (30 mM, pH 10.2), and 0.7 ml of distilled water and 400µl of epinephrine (45 mM). Auto oxidation of epinephrine to adrenochrome was performed in a control tube without the homogenate. Activity was expressed as µmoles/min/mg protein (Kakkar *et al.*, 1984).

Estimation of Catalase

The catalysis of H_2O_2 to H_2O in an incubation mixture adjusted to pH 7.0 was recorded at 254 nm. The reaction mixture contained 2.6 ml of 25 mM potassium phosphate buffer pH 7.0 and 0.1ml of tissue homogenate and was

incubated at 37^0C for 15 minutes and the reaction was started with the addition of 0.1ml of 10 mM H_2O_2. The time required for the decrease in absorbance from 0.45 to 0.4 representing the linear portion of the curve was used for the calculation of enzyme activity. One unit of catalase activity was defined as the amount of enzyme causing the decomposition of µmol H_2O_2/mg protein/min (Abei, 1984).

Estimation of Glutathione reductase

The enzyme activity was determined spectrophotometrically by the decrease in absorbance of NADPH at 340 nm.

$$GSSH + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

The reaction mixture contained 2.1ml of 0.25mM, potassium phosphate buffer pH 7.6, 0.1ml of 0.001M NADPH, 0.2 ml of 0.0165M oxidized glutathione, 0.1ml of BSA(10 mg/ml). The reaction was started by the addition of 0.02 ml of tissue homogenate with mixing and the decrease in the absorbance at 340 nm was measured for

3 minutes against a blank.

Glutathione reductase activity was expressed as µmol NADPH oxidized /min/mg protein (Racker, 1955).

Estimation of peroxidase

Peroxidase activity was measured spectrophotometrically by the following change in absorbance at 460nm due to O-dianisidine oxidation in the presence of H_2O_2 and enzyme. Reaction mixture contained 0.2ml of 15 mM *O*-dianisidine, 0.1ml of tissue homogenate and 2.5 ml of 0.1M potassium phosphate buffer pH 5.0 and were incubated at 37^0C for 15 minutes and the reaction was started with the addition of 0.2 ml of hydrogen peroxide and the absorbance at 460 nm was followed against a blank, spectrophotometrically for about 3-5 minutes at 37^0C. Unit of enzyme activity defined as µmoles of *O*-dianisidine / min (Lobarzewski and Ginalska, 1995).

Estimation of glutathione peroxidase

Glutathione peroxidase activity was measured by the method described by Paglia and Valentine (1967). The reaction mixture consisted of 0.2 ml of 0.4 M phosphate buffer pH 7.0, 0.1 ml of 10 mM sodium azide, 0.1 ml of 0.2 mM hydrogen peroxide, 0.2 ml of glutathione and 0.2 ml of supernatant. The contents were incubated at 37^0C for 10 min. The reaction was arrested by the addition of 0.4 ml 10 % TCA and the absorbance was measured at 340 nm.

DETERMINATION OF NON ENZYMATIC ANTIOXIDANTS

Estimation of reduced glutathione

The method was based on the reaction of reduced glutathione with dithionitrobenzoic acid (DTNB) to give a compound that absorbs at 412nm.

To the homogenate 0.1 ml of 10% TCA was added and centrifuged. About 0.1 ml of supernatant was treated with 0.5 ml of Ellmans reagent (19.8mg of 5, 5'-dithiobis-2-nitro benzoic acid DTNB in 100ml of 0.1% sodium nitrate)

and 3.0 ml of phosphate buffer (0.2M, pH8.0) and the absorbance was read at 412nm. Activity was expressed as µmoles/min/mg protein (Ellman, 1959).

Estimation of lactate dehydrogenase

To 1 ml supernatant, 2 ml of distilled water, 0.2 ml of NAD was added. The reaction mixture was mixed and then incubated at 37°C for 15 min. Briefly after incubation, 1.0 ml of dinitrophenyl hydrazine was added and again allowed to react for 15 min To this add 10 ml of 0.4 N sodium hydroxide and intensity of color developed was read at 440 nm using a blank without supernatant. LDH activity was expressed as µmoles of pyruvate liberated/minute.

Histopathological studies

A portion of liver tissue in each group was fixed in 10% formalin solution, dehydrated in gradually increasing

concentrations of ethanol 50-100%, cleared in xylene and embedded (5 µm) in paraffin for histopathological studies. Heamotoxylin and eosin were used for staining and later the microscopic slides of liver cells were photographed (Haematoxylin and eosin X 100).

Statistical analysis

Statistical analysis of the results was carried out by one-way ANOVA followed by Dunnett's test. Results were expressed as mean±SEM from six rats in each group. P values < 0.05 were considered significant.

6 RESULTS

PHYTOCHEMICAL SCREENING

Phytochemical analysis of powdered leaves of *Desmostachya bipinnata* (L) Stapf. showed the presence of carbohydrates, flavonoids, saponins, glycosides, and tannins (Table 2).

Table 2: Phytochemical Screening

Phytochemicals	Fractions of *Desmostachya bipinnata*			
	PEF	nBF	CF	EAF
Carbohydrates	+	+	+	+
Flavonoids	+	+	+	+
Glycosides	+	+	+	+
Saponins	+	+	+	+
Terpinoids	-	-	-	-
Tannins	+	+	+	+
Alkaloids	-	-	-	-

Effect of the fractions of *Desmostachya bipinnata* Linn. on biochemical parameters in liver slice culture *in vitro*

The liver slices treated with paracetamol at a dose of 15 mM exhibited a significant rise in marker enzymes (viz AST, ALT, ALP) and bilirubin levels when compared to control group ($P<0.01$). The treatment of liver slices with PEF, nBF, CF and EAF of *D.bipinnata* at a dose of 100 µg/ml at two different experimental conditions (viz drug present for 30 min + 2 h and drug present only for 2 h) reduced the marker enzymes and bilirubin levels ($P<0.01$) when compared to the paracetamol control. The liver slices treated with

silymarin also reduced the release of marker enzymes and bilirubin levels (P<0.01) which is similar to the control group. (Table 3).

Table 3. Effect of the fractions of *Desmostachya bipinnata* Linn. on biochemical parameters in liver slice culture *in vitro*

TREATMENT	AST (U/L)	ALT (U/L)	ALP (U/L)	Bilirubin (mg/dl)
Control (only KRH)	87.09±0.49	34.14±0.94	88.4±0.99	0.32±.004
Paracetamol 15(mM)	143.57±0.84[a]	138.25±0.51[a]	220.41±0.42[a]	1.29±.004[a]
PEF-30 min+2 h (100µg/ml)	108.06±1.06[b]	69.5±0.38[b]	96.30±0.20[b]	0.54±0.004[b]
PEF-2 h (100µg/ml)	110.7±1.4[b]	69.1±0.41[b]	99.3±0.20[b]	0.58±0.004[b]
nBF-30 min+2 h (100µg/ml)	109.43±0.58[b]	68.8±0.33[b]	97.7±0.37[b]	0.67±0.01[b]
nBF-2 h (100µg/ml)	110.11±0.68[b]	64.4±0.15[b]	99.08±0.17[b]	0.76±0.005[b]
CF-30 min+2 h (100µg/ml)	109.95±0.52[b]	65.6±0.28[b]	98.5±0.27[b]	0.56±0.006[b]
CF-2 h (100µg/ml)	110.53±0.39[b]	61.8±0.45[b]	98.9±0.23[b]	0.66±0.003[b]
EAF-30 min + 2h (100µg/ml)	103.09±0.35[b]	71.09±0.11[b]	99.2±0.24[b]	0.65±0.002[b]
EAF-30 min +2h (100µg/ml)	106.11±0.23[b]	75.5±0.23[b]	101.13±0.54[b]	0.69±0.003[b]
Silymarin-2 h (100µg/ml)	106.09±0.76[b]	58.3±0.10[b]	90.8±0.25[b]	0.51±0.006[b]

Values are expressed as mean ± SEM; n= 6 in each group.
[a]P <0.01 when compared to control, [b]P <0.01 when compared to paracetamol control. (One way ANOVA followed by Dunnett's test).

Effect of fractions of *Desmostachya bipinnata* Linn. on tissue protein

There was a significant ($P<0.01$) reduction in the total protein level in the paracetamol treated groups when compared to the control. But treatment with PEF, nBF, CF and EAF fractions of *D.bipinnata* significantly ($P<0.01$) elevated the protein level when compared to paracetamol control. The activity produced by the fractions were almost comparable to that of the standard silymarin treated group (Table 4).

Effect of fractions of *Desmostachya bipinnata* Linn. on lipid peroxidation

The end products of lipid peroxidation namely malondialdehyde (MDA) and lipid hydroperoxides (LH) was found to be significantly ($P<0.01$) higher in paracetamol treated groups. But treatment with the fractions of *D.bipinnata* significantly decreased ($P<0.01$) the MDA and LH levels and were comparable with the silymarin treated group(Table 4).

Effect of fractions of *Desmostachya bipinnata* on lactate dehydrogenase

Liver slices released more LDH in to the medium in the presence of paracetamol when compared to control ($P<0.01$). But when the liver slices were treated with various fractions of *D.bipinnata,* the release of LDH in to the medium was significantly ($P<0.01$) reduced. The liver slices treated with silymarin also reduced the release of LDH ($P<0.01$) which is similar to the control group (Table 4).

Table 4. Effect of the fractions of *Desmostachya bipinnata Linn.* on tissue protein, MDA, LH and LDH in liver slice culture *in vitro*

TREATMENT	TP (mmoles / min/mg tissue)	MDA (µmoles/min/ mgprotein)	LH (µmoles/ min/mg protein)	LDH (µmolepyruvate/ min/mg protein)
Control (only KRH)	99±2.12	0.110±0.001	0.205±0.001	6.99±0.123
Paracetamol 15(mM)	88±1.23[a]	0.389±0.002[a]	0.451±0.006[a]	16.12±0.320[a]
PEF-30 min+ 2 h (100µg/ml)	94±0.56[b]	0.138±0.003[b]	0.193±0.001[b]	8.12±0.230[b]
PEF-2 h (100µg/ml)	93±1.20[b]	0.132±0.002[b]	0.205±0.002[b]	8.32±0.412[b]
nBF-30 min+ 2 h (100µg/ml)	95±2.12[b]	0.136±0.001[b]	0.196±0.001[b]	6.54±0.148[b]
nBF-2 h (100µg/ml)	93±2.13[b]	0.133±0.001[b]	0.185±0.003[b]	8.69±0.198[b]
CF-30 min+2 h (100µg/ml)	96±0.132[b]	0.135±.001[b]	0.195±.0.001[b]	8.73±0.013[b]
CF-2 h (100µg/ml)	94±1.48[b]	0.130±0.002[b]	0.187±0.001[b]	8.80±0.120[b]
EAF-30min+2h (100µg/ml)	95±1.01[b]	0.132±0.001[b]	0.189±0.001[b]	8.99±0.133[b]
EAF-2h (100µg/ml)	91±1.11[b]	0.129±0.005[b]	0.199±0.004[b]	8.95±0.154[b]
Silymarin-2 h (100µg/ml)	91±0.12[b]	0.151±0.004[b]	0.216±0.009[b]	8.45±0.127[b]

Values are expressed as mean ± SEM; n= 6 in each group.

[a]P <0.01 when compared to control, [b]P <0.01 when compared to paracetamol control. (One way ANOVA followed by Dunnett's test).

Effect of the fractions of *Desmostachya bipinnata* Linn. on enzymatic and non enzymatic antioxidants

The levels of tissue enzymatic antioxidants namely SOD, CAT, GSSH, GPx and the non enzymatic anti oxidant GSH in paracetamol group was found to be significantly ($P<0.01$) lower compared to the normal control. Whereas treatment of liver slices with the various fractions (viz PEF, nBF, CF and EAF) of *D.bipinnata* elevated the enzymatic and non enzymatic antioxidants. The antioxidant activity of the fractions were comparable with that of the standard drug silymarin. (Table 5).

Table 5. Effect of the fractions of *Desmostachya bipinnata* Linn. on enzymatic and non enzymatic antioxidants

TREATMENT	SOD	CAT	GSH	GSSH	GPx	Px
Control (only KRH)	1.142±0.38	278±0.030	2.87±0.307	3.66±0.193	3.022±0.05	1.27±0.04
Paracetamol 15(mM)	0.589±0.41[a]	231±0.004[a]	2.15±0.012[a]	2.30±0.049[a]	2.55±0.03[a]	0.771±0.014[a]
PEF-30 min+2 h (100µg/ml)	0.869±0.012[b]	272±0.321[b]	2.69±0.01[b]	2.91±0.020[b]	2.61±0.039[b]	1.29±0.015[b]
PEF-2 h (100µg/ml)	0.762±0.003[b]	270±0.012[b]	2.54±0.031[b]	2.85±0.027[b]	2.59±0.05[b]	1.19±0.032[b]
nBF-30 min+2 h (100µg/ml)	0.812±0.069[b]	274±0.004[b]	2.48±0.01[b]	2.75±0.032[b]	2.50±0.01[b]	1.10±0.008[b]
nBF-2 h (100µg/ml)	0.809±0.0120[b]	272.±0.003[b]	2.37±0.001[b]	2.79±0.033[b]	2.42±0.02[b]	1.09±0.009[b]
CF-30 min+2 h (100µg/ml)	0.791±0.008[b]	276±0.009[b]	2.36±0.012[b]	2.76±0.040[b]	2.38±0.042[b]	1.08±0.018[b]
CF-2 h (100µg/ml)	0.782±0.001[b]	273±0.003[b]	2.34±0.033[b]	2.80±0.023[b]	2.42±0.03[b]	1.07±0.014[b]
EAF-30min+2h (100µg/ml)	0.778±0.001[b]	276±0.005[b]	2.44±0.035[b]	2.89±0.024[b]	2.43±0.05[b]	1.07±0.015[b]
EAF -2h (100µg/ml)	0.798±0.00[b]	272±0.006[b]	2.49±0.032[b]	2.95±0.012[b]	2.45±0.06[b]	1.11±-.001[b]
Silymarin-2 h (100µg/ml)	0.898±0.012[b]	281±0.004[b]	2.68±.0.016[b]	3.06±0.112[b]	2.81±0.11[b]	1.32±0.020[b]

Values are expressed as mean ± SEM ; n= 6 in each group.

CAT = mmoles/min/mg protein, GPx = µmoles/min/mg protein, GSH = µmoles/min/mg protein, SOD= µmoles/min/mg protein,

Px = µmoles/min/mg protein, and GSSH = µmoles/min/mg protein.

[a]P <0.01 when compared to control, [b]P <0.01 when compared to paracetamol control. (One way ANOVA followed by Dunnett's test).

HISTOPATHOLOGICAL STUDIES

Histopathological results of the liver slices are shown from fig.8 to fig.14. Fig.9 represents the liver section of control group showing normal hepatocytes, portal triads, central veins and sinusoids. Fig. 10 represents the liver section of paracetamol treated group showing marked congestion of central veins and sinusoids with periportal inflammation extending to the lobules. Fig. 11 represents the liver section of PEF treated group showing mild to moderate periportal and lobular inflammation with mild congestion of the sinusoids. Fig. 12 represents the liver section of nBF treated group showing mild periportal inflammation. Fig. 13 represents the liver section of CF treated group showing mild to moderate periportal and lobular inflammation with mild congestion of the sinusoids. Fig. 14 represents the liver section of EAF treated group showing marked congestion of few central veins and sinusoids with periportal inflammation. Fig 15 represents the liver section of silymarin treated group showing normal portal triad, central vein and radiating cords of hepatocytes.

Fig 9: Liver section of Control group

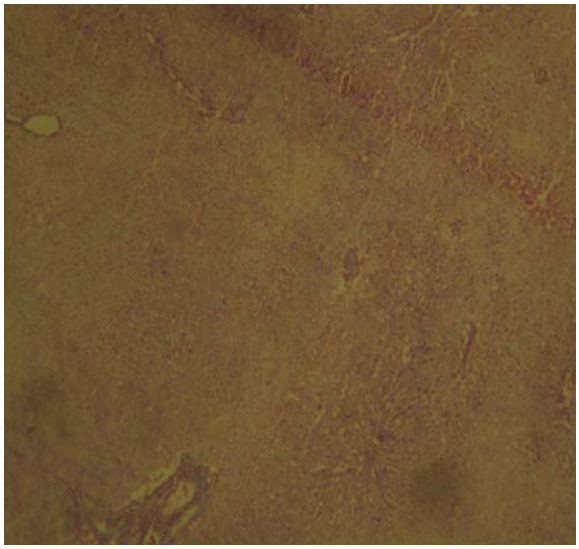


Fig 10: Liver section of Paracetamol treated group

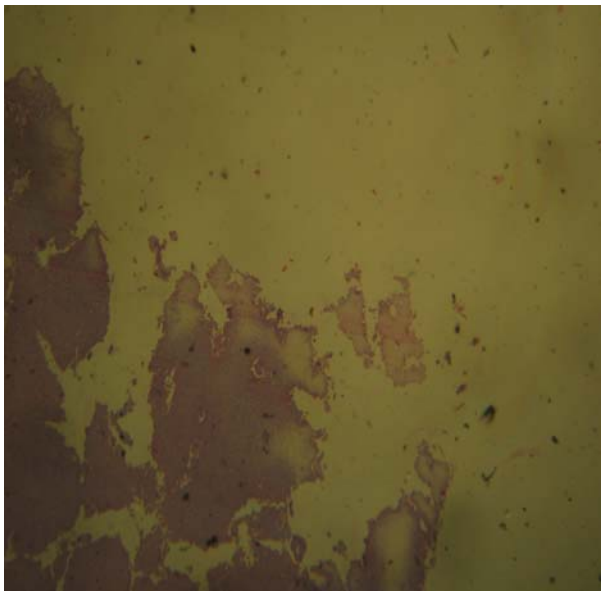


Fig 11: Liver section of group treated with Petroleum ether fraction and paracetamol

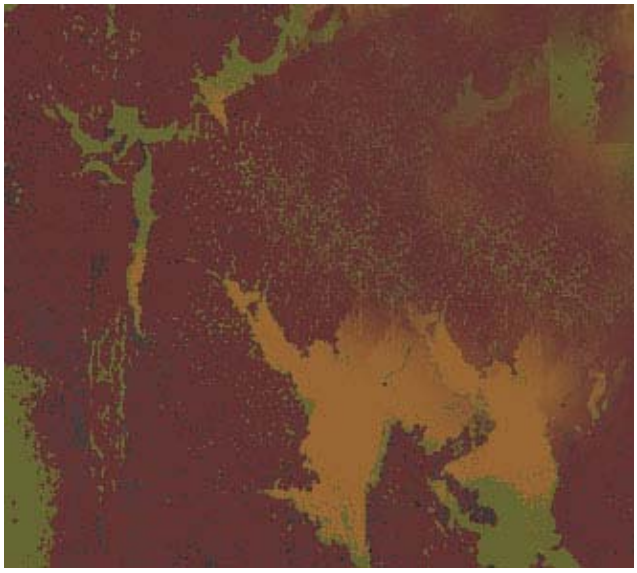


Fig 12: Liver section of group treated with n-Butanol fraction and Paracetamol

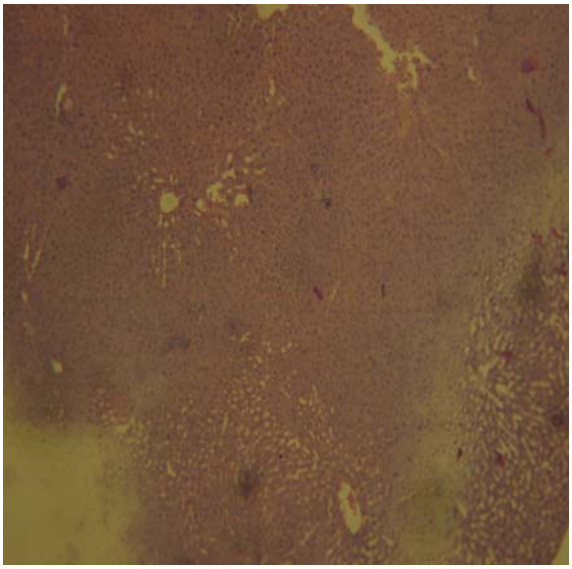


Fig 13: Liver section of group treated with Chloroform fraction and Paracetamol

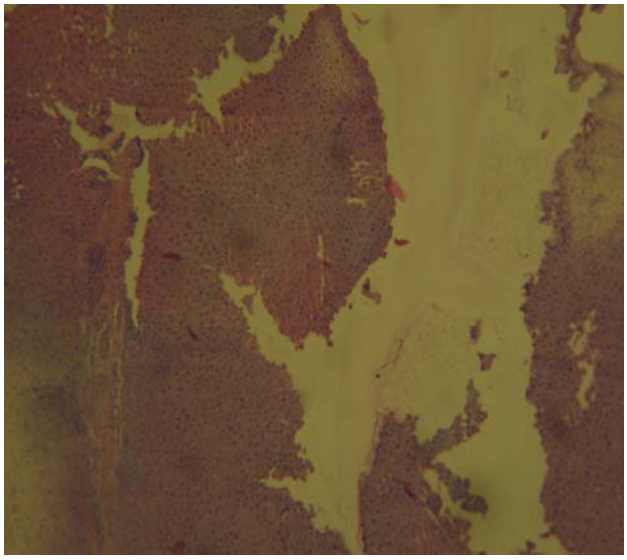


Fig 14: Liver section of group treated with Ethyl acetate fraction and Paracetamol

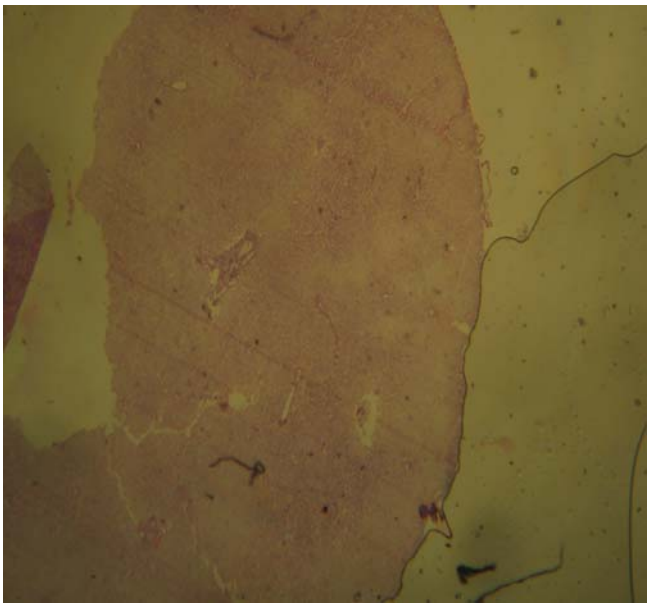
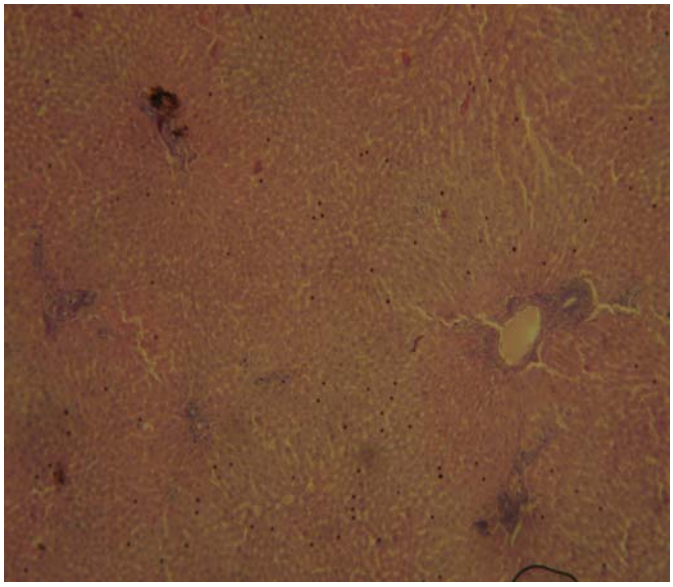


Fig 15 :Liver section of group treated with silymarin treated group and Paracetamol



7 DISCUSSION AND CONCLUSION

The present thesis entitled "Protective effect of *Desmostachya bipinnata* Linn. against paracetamol-induced hepatic damage" deals with the exploration of pharmacological and phytochemical screening of the selected Indian medicinal plant *Desmostachya bipinnata* belonging to the family Poaceae, which is traditionally useful in thirst, astma, Jaundice, skin eruption, dysentery, uropathy etc. (Kirtikar and Basu, 1987).

The liver performs many functions vital to the health of the organism. The liver transforms and excretes many drugs and toxins. These substances are frequently converted in to inactive form by reactions that occur in the hepatocytes. Certain enzymes in the endoplasmic reticulum catalyze the conjugation of many compounds.

Transformations that occur in the liver renders many drugs water soluble and they are readily excreted by kidneys. The liver is the target organ for drug- induced lesions. The physiological response to injury results in a variety of lesions such as necrosis, cholestasis, steatosis, inflammation and fibrosis. Paracetamol-induced hepatotoxicity serves as an excellent model to study the molecular, cellular and morphological changes in the liver (Waugh and Grant, 2001; Zhang , 2002).

Liver slice culture is therefore an *in vitro* technique that offers the advantage of *in vivo* situation and hence is a more suitable model for the experimental analysis of hepatotoxic events (Naik *et al*., 2004).

Paracetamol is metabolized primarily in the liver where 60-90% is converted to inactive compounds by conjugation with sulfate and glucuronide and then excreted by the kidney. Acetaminophen is mainly metabolized by cytochrome P-450 to form an electrophilic metabolite N-acetyl *p*-benzo quinonimine (NAPQI), which is primarily inactivated by conjugation with glutathione. At high doses, the detoxification pathway becomes saturated and the intermediate metabolite accumulates and causes liver damage by covalent binding to tissue molecules. Acetaminophen hepatotoxicity appears to be critically dependent on the depletion of cellular glutathione (Arnaiz *et al.,* 1995).

Historically plants have been used in folk medicine to treat various diseases and are rich sources of natural antioxidants. *Desmostachya bipinnata* Linn. belonging to the family Poaceae is

widely distributed in India. The major constituents present in this plant are glycoside, flavanoids, saponins, tannins, carbohydrates. (The Wealth of India, 1976). *Desmostachya bipinnata* is used in indigenous medicine as asthma, thirst, jaundice, disease of the blood, skin eruption, dysentery, uropathy, strangury, vesical calculi (Kiritikar and Basu, 1987). Based on the above literatures the present study was carried out to evaluate the protective effect of the various fractions of *Desmostachya bipinnata* Linn. against paracetamol-induced hepatotoxicity using liver slice culture *in vitro*.

Incubation of paracetamol (15 mM) caused significant ($P<0.01$) elevation in the transaminases and ALP activities compared to the normal control. Bilirubin and enzyme levels are the most sensitive markers employed in the diagnosis of hepatic damage. The elevation of transaminase and ALP indicated the necrosis of hepatocytes that results in the leakage of transaminases and the elevation of ALP from a possible cholestasis. A significant decrease in the transaminases and ALP activities in the groups treated with the various fractions of *Desmostachya bipinnata* Linn. group demonstrated its hepatoprotective effect.

Excess levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) can attack biological molecules such as DNA, protein and phospholipids, which leads to lipid peroxidation, nitration of tyrosine and depletion of antioxidant enzymes (Yen *et al.,* 2007). Superoxide dismutase is an enzymatic antioxidant which scavenges the superoxide anion and converts it in to hydrogen peroxide, hence

diminishing the toxic effect caused by this radical. Catalase decomposes hydrogen peroxide and protects the tissue from highly toxic hydroxyl radicals. Therefore, due to the accumulation of superoxide radical and hydrogen peroxide there will be a reduction in the activity of these enzymes and may result in deleterious effect (Husain, 2002; Murugesh *et al.,* 2005). The treatment of liver slices with the fractions of *Desmostachya bipinnata* Linn. inhibited the deleterious effect of free radicals and restored the antioxidant levels.

Overdose of paracetamol elevates malondialdehyde (MDA) and lipid hydroperoxides (LH), the byproducts of lipid peroxidation. MDA is used as an indicator of tissue damage by a series of chain reactions (Husain, 2002 ; Gupta *et al.,* 2004 ; Wendel 1979). The increase in MDA and LH levels in liver slice treated with paracetamol suggests the enhanced lipid peroxidation, leading to tissue damage and failure of antioxidant defence mechanism to prevent formation of excessive free radicals. Catalase, which acts as preventative antioxidant plays an important role in protection against the deleterious effects of lipid peroxidation. But, treatment with the fractions of *Desmostachya bipinnata* Linn. decreased the elevated MDA & LH levels.

LDH is a cytosolic enzyme mainly present in periportal hepatocytes and released when the cells are lysed by hepatotoxins. The amount of enzyme released is directly proportional to the extent of damage caused to the cells. Paracetamol treated liver slices released 2.5 times more enzymes into the medium than untreated cells over a period of 2 h. The fractions of *Desmostachya bipinnata* added to liver

slices with paracetamol lowered the enzyme level and prevented its leakage into the medium (Naik *et al.,* 2004).

Glutathione peroxidase has a well established role in protecting cells against oxidative injury. GPx is non specific for hydrogen peroxide and lack of this substrate specificity extends a range of its substrates from hydrogen peroxide to organic hydroperoxides (Rukkumani *et al.,* 2004).

GSH is an intracellular reductant and plays major role in catalysis, metabolism and transport. It protects cells against free radicals, peroxides and other toxic compounds. GSH depletion increases the sensitivity of cells to various aggressions and also has several metabolic effects. Liver slice treated with paracetamol showed a significant decrease in the level of the non enzymatic antioxidant, glutathione due to the inhibition of its protective effect against lipid peroxidation. Treatment with the fractions of *Desmostachya bipinnata* Linn. elevated the activity of glutathione due to its protective mechanism against paracetamol damage.

Silymarin by its stabilizing action on the plasma membrane has been shown to normalize paracetamol induced biochemical parameters in the liver. It has protective effect on paracetamol induced lipid peroxidation and glutathione depletion. Silymarin exhibits its hepatoprotective action either by preventing hepatic cell necrosis or by inducing hepatic cell regeneration.

In conclusion, the results of our study clearly demonstrated that the fractions of *Desmostachya bipinnata* Linn. exhibited potent hepatoprotective activity against paracetamol- induced hepatic

damage. This may be due to their antioxidant and free radical scavenging properties. Further studies are needed to isolate and purify the active principles involved in the hepatoprotective efficacy of the leaves of *Desmostachya bipinnata* Linn.

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